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The expression of heme oxygenase-1 in the gastrointestinal tract

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The Expression of Heme Oxygenase-1
in the Gastrointestinal Tract

Dr Mark Feeney MA, MB BCh, MRCP

A thesis submitted for the degree of MD

University of Bath

2005

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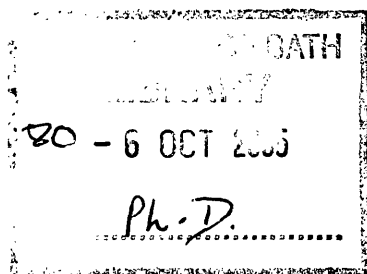
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(iii) Abstract

Background: Heme oxygenase (HO) functions in the rate-limiting step in the metabolism of heme to form biliverdin, carbon monoxide and free iron (Fe^{2+}). HO has an inducible form HO-1 and constitutive forms HO-2 and HO-3. HO-1 is highly conserved and essential for life. It has been suggested that in addition to the metabolism of heme, HO-1 may have anti-oxidant and anti-inflammatory roles. Induction of HO-1 in pulmonary, hepatic and brain tissues has been shown to reduce damage under ischaemic and inflammatory conditions.

Hypothesis: Loss of appropriate regulation of HO-1 may cause a reduction in HO-1 levels resulting in an increase in reactive oxygen species in the gastrointestinal tract leading to the development of uncontrolled inflammation. This reduction in HO-1 may play a role in the development of inflammatory bowel disease, colorectal cancer and Barrett's oesophagus.

Aims and Methods: Immuno-staining was used to compare the expression of HO-1 and HO-2 in normal colon, inflammatory bowel disease (IBD) and colorectal cancer. A similar comparison was made between biopsies from normal oesophagus and Barrett's oesophagus. In all cases the intensity of staining was scored in both the lamina propria and the epithelial cell layer. Western blotting was used to assess HO-1 protein production in the colon cell line HT29.

Results: HO-1 was expressed in normal colon and oesophageal tissue in the epithelial cell layer and in some of the inflammatory cells of the lamina propria. HO-1 was also expressed in the blood vessel walls and the parasympathetic ganglia in the submucosa in healthy colon. There was a significantly increased expression in the lamina propria in IBD, the epithelial layer in colorectal cancer and in both the lamina propria and epithelial cells of Barrett's oesophagus. The colonic cell line HT-29 expressed HO-1 and the expression was further increased by exposure to ultra-violet light.

Conclusions: HO-1 is increased in conditions of inflammation or cancer, suggesting an appropriate physiological response to reactive oxygen species rather than a deficiency state in HO-1.

(iv) Abbreviations

Ab	Antibody
APS	Ammonium Persulphate
5-ASA	5-aminosalicylate
BSA	Bovine Serum Albumin
CD	Crohn's Disease
COX	Cyclo-oxygenase
CRC	Colorectal carcinoma
DMSO	Dimethylsulphoxide
DSS	Dextran Sodium Sulphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescent Activated Cell Sorting
FBS	Foetal Bovine Serum
GI	Gastrointestinal
HBSS	Hank's Balanced Salt Solution
HO	Heme-oxygenase
IBD	Inflammatory Bowel Disease
IFN	Interferon
IL	Interleukin
IMS	Industrial methylated spirit
JNK	<i>c-jun</i> N-terminal Kinase
kb	Kilo-base
kDa	Kilo-Dalton
LPS	Lipopolysaccharide
MAP Kinase	Mitogen Activated Protein Kinase
mRNA	Messenger Ribonucleic acid
MQ	Milli Q (water)
MWt	Molecular Weight
MWU	Mann Whitney U
NFκB	Nuclear Factor κ B

NO	Nitric Oxide
NOS2	Inducible Nitric Oxide Synthase
NSAID	Non-steroidal Anti-inflammatory Drug
PBS	Phosphate buffered saline
PG	Prostaglandin
PI 3-K	Phosphatidylinositol 3-Kinase
PMN	Polymorphonuclear Neutrophil
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamids Gel Electrophoresis
SSPE	Saline-Sodium Phosphate-EDTA Buffer
TBS	Tris Buffered Saline
TEMED	N, N, N', N'-tetramethylethylene Diamine
TGF	Transforming Growth Factor
Th1	T Helper 1
Th2	T Helper 2
TNBS	Trinitro Benzene Sulphonic (Acid)
TNF	Tumour Necrosis Factor
UC	Ulcerative Colitis
UV	Ultraviolet

1 INTRODUCTION

1.1 Normal structure of the gastro-intestinal tract

The gastrointestinal tract (GI tract) is a muscular tube that stretches from the mouth to the anus. There is a common pattern to the structure along its length, which consists of 4 layers. The layers from the luminal surface inwards are: the mucosa, the submucosa, the muscularis and the adventitia or serosa. (Figure 1, Wheater et al 1979). The muscular parts of the tract are relatively similar throughout the gut; however the character and function of the mucosa vary along its length. There are several points at which there is an abrupt change in the type of mucosa: at the gastro-oesophageal junction, the gastro-duodenal junction, ileo-caecal junction and the recto-anal junction. In other parts of the GI tract such as the small bowel there are gradual changes in the character of the mucosa.

The mucosa consists of 3 layers: (1) the epithelium lines the lumen, (2) the lamina propria which is made up of supportive connective tissue and is the site of many inflammatory cells and (3) the muscularis mucosae which is a very thin layer of smooth muscle capable of causing small scale movements of the mucosa and offering some resting tone which holds the mucosal folds in place (see Figures 2 and 3).

The submucosa is a layer of connective tissue that supports the structure of the mucosa and contains the blood vessels, nerves and lymphatics. The muscularis is made up of two smooth muscle layers, the inner being circular and the outer

longitudinal. The co-ordinated contraction of these smooth muscle layers gives rise to the peristaltic waves that lead to the progression of the luminal contents along the GI tract. The adventitia is the outer layer of the GI tract and is made up of connective tissue: within this layer are the larger blood vessels and nerves. Post-ganglionic parasympathetic nerves supply the mucus glands and the smooth muscle of the muscularis mucosae and arise from the parasympathetic plexus within the submucosa (Meissner's plexus). The parasympathetic nerves supplying the larger circular and longitudinal muscle layers of the muscularis arise from the myenteric plexus which is usually found between the circular and surrounding longitudinal layers.

Figure 2 and 3 show haematoxylin and eosin staining of normal terminal ileum and colon. This is the standard staining technique used for histological analysis of tissue. The essential structural elements of the mucosa are labelled within figure; there is a large lymphoid aggregate and many villi cut in cross-section (Figure 2). Epithelial cells line the luminal surface and just below this is the lamina propria containing inflammatory cells and stromal components. Figure 3 shows the structure of the normal colonic mucosa. There are no villi in the colon; the crypts lined with epithelial cells are clearly seen, cut both in cross-section and longitudinal section. Beneath the lamina propria the muscularis mucosae is marked. Deep to the muscularis mucosae is the submucosa and blood vessels are marked in this region.

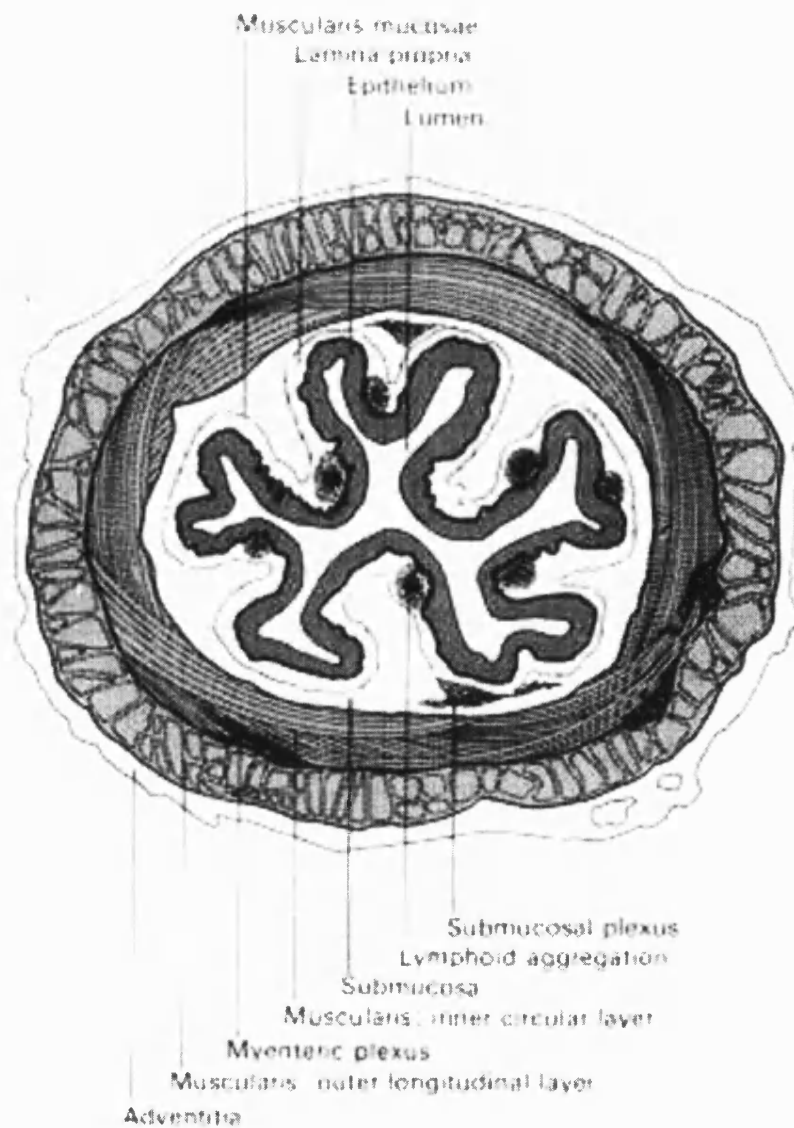


Figure 1 The general structure of the gastrointestinal tract in cross section.

From Wheater et al (1979) Functional Histology a Text Colour Atlas.
Churchill Livingstone

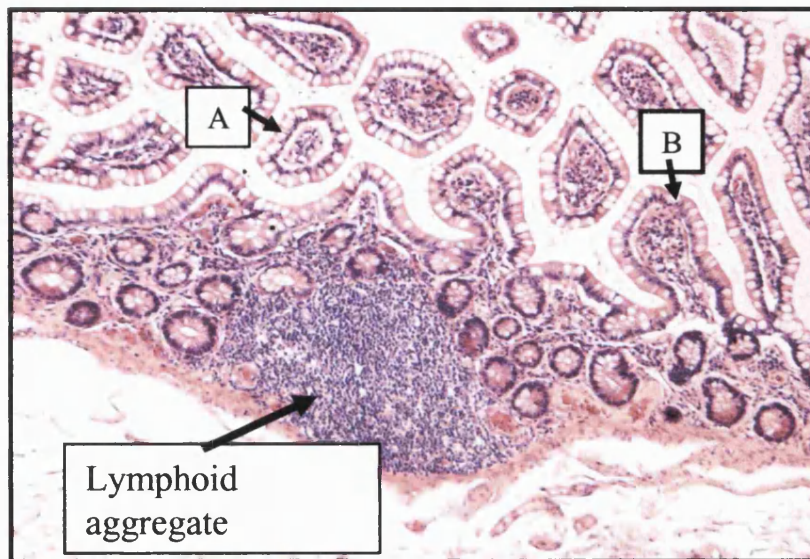


Figure 2 Normal terminal ileum stained with Haematoxylin and Eosin
 (A) Villus in cross-section, (B) Villus in longitudinal section.
 (original magnification of 128 x)

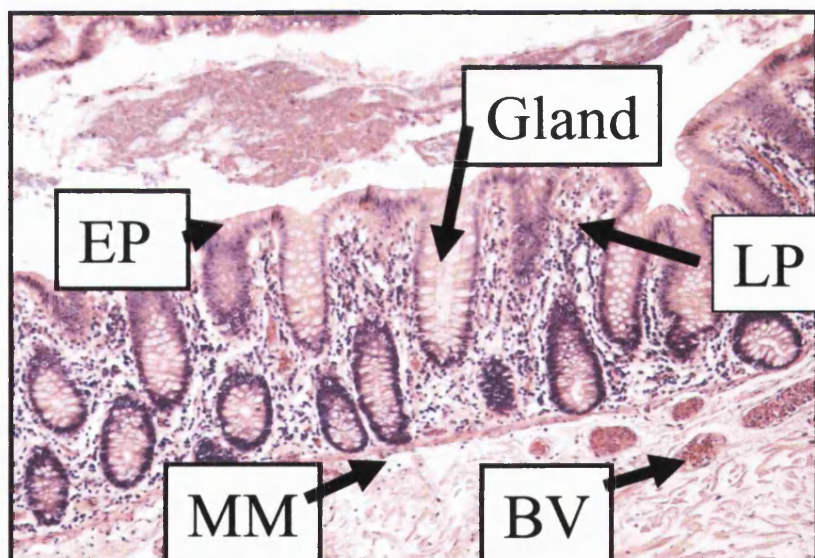


Figure 3 H + E slide to identify structures within the normal colonic mucosa: EP epithelial cell, MM the muscularis mucosae, BV blood vessel in the the submucosa, LP the lamina propria (original magnification of 128 x)

1.2 Inflammatory Bowel Disease

Crohn's disease and ulcerative colitis are diseases of chronic inflammation of the gut, together they make up inflammatory bowel disease. Their aetiology remains unclear but is influenced by both genetic and environmental factors. Combined incidence rates in the Western world are in the order of 4 –10 new cases per 100,000 per year and prevalence is between 40 and 100 per 100,000 of population. These conditions are more common in the developed world, some of the highest rates being found in Scandinavia. The major defining feature of UC is that inflammation is confined to the colon, whereas CD inflammation can occur in any part of the gut from the mouth to the anus.

1.2.1 Characteristic histological features of IBD

In UC acute and chronic inflammation are confined to the mucosa involving polymorphonuclear leukocytes and mononuclear cells. Other classical histological features include crypt abscesses; mucosal gland distortion and goblet cell depletion (see Figure 4). The inflammatory change almost always involves the rectum and variable amounts of the more proximal colon. In CD the inflammatory change can involve the full thickness of the bowel wall in any part of the gut. Classical CD defining lesions include granuloma formation and aphthous ulceration. Diseased areas of gut may be surrounded by normal tissue.

1.2.2 Clinical presentation and diagnosis

Both UC and CD are characterised by episodes of uncontrolled inflammation affecting the gut wall. UC tends to present with a change in bowel habit,

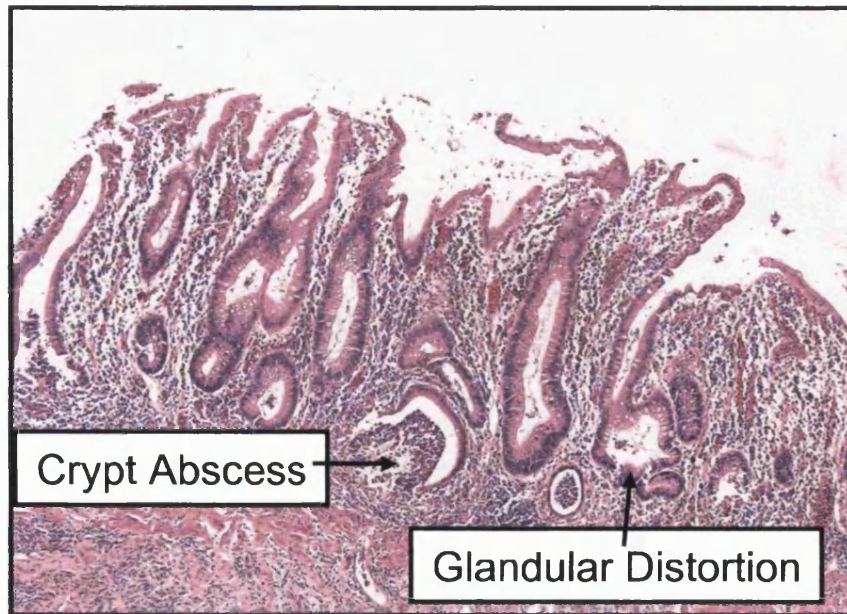


Figure 4 H+E stained colonic biopsy from a patient with UC. This slide shows a crypt abscess, glandular distortion, there is also marked inflammatory cell infiltrate throughout the lamina propria Original magnification 128x.

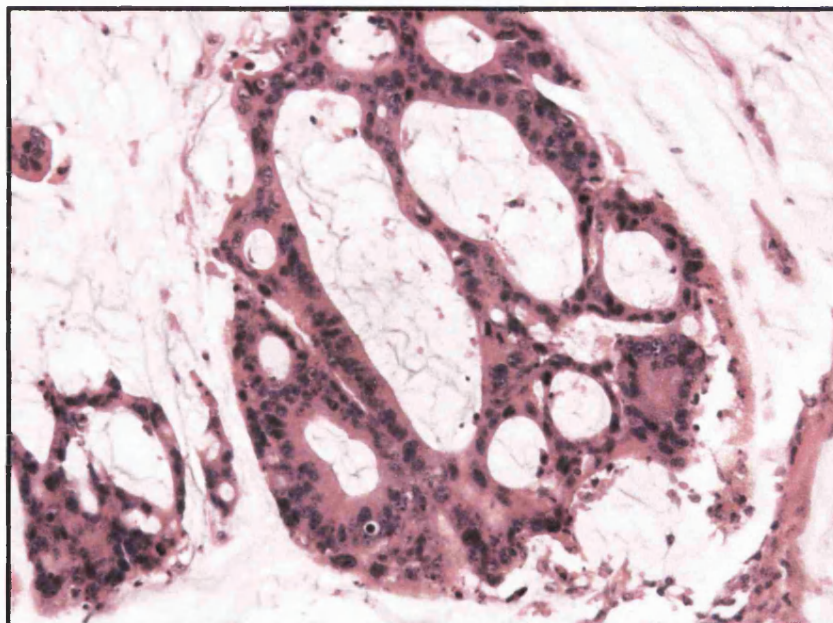


Figure 5
Hemeatoxylin and eosin slide of a mucinous colonic carcinoma
original magnification 128x.

producing a watery, bloodstained stool, of increased frequency. This may be accompanied by lower abdominal pain. CD most commonly affects the ileo-caecal region i.e. the end of the small bowel as it joins the colon, this may result in abdominal pain, weight loss and possibly a change in bowel habit. If CD affects the colon then it may present in a similar fashion to UC and the diagnosis will then depend on the distribution of the inflammatory changes and on histology of colonic biopsy specimens. When CD affects other parts of the gastro-intestinal tract it may present in a less obvious way. Inflammation caused by CD may lead to some regions of the bowel becoming narrowed to form a stricture. Strictures can give rise to postprandial pain. CD of the upper gut can also give rise to nausea, weight loss and epigastric pain.

Diagnosis of IBD depends on histological features of biopsy material and the distribution of the inflammation. Endoscopic examination of the colon (colonoscopy) is the primary diagnostic tool in colonic IBD. Using the video endoscope it is possible to view the mucosal lining in real time and take tissue specimens (biopsies). The small bowel is relatively inaccessible to conventional endoscopic techniques and diagnosis often depends on radiological tests, which can indirectly demonstrate which parts of the gut are affected by inflammation.

1.2.3 Medical therapy and IBD

Although UC and CD were first clearly defined in the 1930s, it was not until the 1940s that effective medical therapy became available. Surgery for severe inflammation was not always successful and IBD had a significant mortality. In the 1940s it was discovered that sulfasalazine, a drug used to treat arthritis, also

reduced bowel inflammation (Svartz, 1954). This fortuitous observation led to the development of a family of agents designed around the 5-aminosalicylate structure. In the 1950s, following the work of Truelove and colleagues in Oxford it was shown that severe inflammation in IBD could be effectively treated with systemic steroids, usually prednisolone (Truelove, 1960). 5-aminosalicylic acid agents and steroids formed the mainstay of medical therapy for IBD over the next 40 years.

Recurrent bouts of inflammation necessitating repeated steroid treatments can lead to long-term steroid side effects such as osteoporosis, muscle wasting, adrenal suppression, diabetes mellitus, hypertension and skin fragility. In the last decade there has been renewed interest in other forms of immuno-suppression, most notably the use of azathioprine. Used as maintenance therapy, once remission has been achieved, it reduces the likelihood of further flares of inflammation and avoids the problems associated with steroid therapy. There is still an essential role for the use of short courses of steroids to induce remission in IBD. Maintenance of remission can then usually be achieved by continuing azathioprine or high dose 5 aminosalicylate agents. Other immunosuppressant agents have been tested: in acute UC intravenous cyclosporin can delay the need for surgery but is not thought to greatly change the overall outcome. Tacrolimus has proved to be disappointing. Some therapies have been shown to be particularly useful in CD. Antibiotics such as metronidazole and ciprofloxacin have been shown to improve CD especially peri-anal disease. This observation supports the role of bacteria in the aetiology of CD, which will be further

discussed below. Methotrexate and thalidomide have also proved to be useful second-line therapies in CD.

1.2.4 Advent of biological therapies

In the last 10 years there have been great advances in the understanding of the control of inflammation. This has led to the design of drugs targeted at individual cytokines. The best example is the action of a chimeric antibody specific to Tumour Necrosis Factor alpha (TNF α), a therapy originally developed to treat rheumatoid arthritis. When given as an intravenous infusion over 3 occasions, it achieved a clinical response in 81% of cases and remission in 48% (Targan, Hanauer et al. 1997). It has been found to be particularly successful when treating fistulating CD.

1.2.5 Natural history of IBD

Patients with severe UC have a 25- 40% lifetime rate of colectomy. Surgery is reserved for 4 types of patients with IBD. Firstly, for patients who fail to achieve remission with medical treatment. Secondly, those who present with the toxic megacolon, a complication of severe colonic inflammation in which a sudden dilatation of the colon occurs risking perforation and death. Thirdly, fibrotic strictures can develop after years of grumbling inflammation; these do not respond to anti-inflammatory medication and if they are causing symptoms of bowel obstruction will require surgery to restore a functioning lumen. Finally, surgery may be required for those patients who develop colorectal carcinoma as a complication of a long duration of colitis. Most patients who require surgery for CD will need a second operation within 20 years. The risk of further surgery in

CD can be reduced by maintenance azathioprine (Travis 2001) and the cessation of smoking (Cosnes, Beaugerie et al. 2001). Unlike for UC, surgery in CD can never be considered curative; the disease is not confined to the colon and therefore can recur elsewhere.

1.2.6 Pathogenesis of IBD

1.2.6.1 Genetics and IBD

Twin studies show that there is a major genetic influence in IBD. The chance of both twins having CD is much higher for monozygotic compared to dizygotic twins. Family studies show that first-degree relatives of a patient with IBD are tenfold more likely to develop IBD than normal subjects. Within the last couple of years exciting discoveries linking CD to a particular gene defect have been made. Mutations of the NoD2 (nucleotide oligomerisation domain) gene on chromosome 16 have been linked to an increased risk of CD (Hugot, Chamaillard et al. 2001; Ogura, Bonen et al. 2001). Mutations in this region occur in 23 % of patients with CD compared to only 7% of normal subjects. The NoD2 gene codes for a receptor specific to the lipopolysaccharide of bacterial cell walls, and may have a role preventing bacterial invasion of the gut wall. Activation of this receptor is thought to result in the up-regulation of NF κ B. The most common mutation is a frame shift change, which results in truncation of the last 3% of the protein and a decrease in the LPS-induced activation of NF κ B. This is somewhat surprising, as this would appear to reduce any inflammatory response to bacteria. Although this defect remains incompletely explained, it does represent the first genetic abnormality identified which has a functional significance related to IBD. More recently the phenotype of CD associated with the mutations on the NoD2 gene

associated with ileal disease has been characterised by two groups (Ahmad, Armuzzi et al. 2002; Cuthbert, Fisher et al. 2002). It may be that future genetic analysis will be able to identify specific subgroups within IBD, giving prognostic information to guide the selective use of medical or surgical therapy.

1.2.6.2 Auto-immune mechanisms and IBD

It has been suggested that several of the features of UC could be accounted for by an auto-immune process directed against a self antigen such as mucin, goblet cells or colonocytes (MacDonald, Monteleone et al. 2000). It has long been recognised that the anti-neutrophilic cytoplasmic antibody is associated with UC, although its levels do not correlate with disease activity (Roozendaal, Pogany et al. 1999). Other significant antigens have proved to be difficult to identify. It is now thought that the most significant drive to inflammation in Crohn's disease is the presence of foreign antigens rather than an auto-immune process.

1.2.6.3 Role of intestinal epithelial cells

A single layer of colonic epithelial cells is the only barrier between the gut lumen and the underlying mucosal immune system. The colonic epithelial cells are exposed to the luminal contents of the gut (for example, bacteria) and are capable of producing cytokines (such as MCP-1), which then can influence the behaviour of sub-epithelial lymphoid tissue. In this way they may act as early warning sentinels for the immune system. Interesting data produced by Ward's group at Bath have shown that the production of COX-2 by the intestinal epithelial cells can be inhibited/down-regulated by the Th2 cytokines IL-4, IL-10 and IL-13 via a PI3K dependent mechanism (Weaver, Russo et al. 2001).

1.2.6.4 Environmental factors and IBD

Sheltered Child Hypothesis

Environmental conditions particularly in the early years of life appear to influence the risk of developing IBD. A study of the availability of a hot water tap in the childhood home showed that individuals who had access to hot water had a fivefold increased odds ratio of developing CD compared to those without (Gent, Hellier et al. 1994). The study population had been born in the 1950s and 1960s and so not all had access to such amenities. Other studies have shown a reduced rate of *Helicobacter pylori* seropositivity in CD patients compared to UC and to individuals without IBD. *Helicobacter pylori* is transmitted by the faeco-oral route and so can be used as a surrogate marker of poor hygiene. Again, it can be argued that the low seropositivity of *H.pylori* associated with CD shows a link between good childhood hygiene and CD (Feeney, Murphy et al. 2002, Table 1). It has been suggested that such conditions may delay the exposure to previously prevalent childhood infections. It is possible that when encountered later in life such infections could trigger inappropriate inflammation. This could occur directly by affecting the GI tract or as a result of the development of an antibody raised against an infective organism that inappropriately cross-reacts with a host protein. This theory could account for the rising prevalence of CD in the Western world over the last 50 years. Interestingly over this period the prevalence of UC has remained relatively unchanged (Logan, 1998).

A case-control study of childhood environmental risk factors for the development of inflammatory bowel disease.

Feeney MA, Murphy F, Clegg AJ, Trebble TM, Sharer NM and Snook JA

European Journal of Gastroenterology and Hepatology 2002.

Table 1

Odds ratios (with 95% confidence intervals)

	Crohn's Disease	UC
Helicobacter Pylori	0.18 (0.06-0.52)*	0.91 (0.38 - 2.16)
Appendectomy	0.53 (0.16 -1.73)	0.05 (0.01-0.51)#
Childhood eczema	2.81 (1.23- 6.42)+	1.83 (0.73- 4.59)

(* P =0.002, # p=0.011, + p=0.014)

Smoking and IBD

Smoking seems to alter the risk of IBD and to affect which type of disease an individual develops. In many studies an excess risk of CD has been demonstrated in smokers and of UC in ex-smokers (Fiocchi 1998; Bridger, Lee et al. 2002). Smoking cessation can reduce the risk of further surgery in CD (Cosnes, Beaugerie et al. 2001). Paired siblings both suffering from IBD but with a different smoking history demonstrate that smoking may determine which phenotype of disease develops in a genetically susceptible individual. Of sibling pairs discordant for both type of IBD and smoking history those who smoked were far more likely to have CD than UC (this pattern was seen in 21 out of 23 such sibling pairs) (Bridger, Lee et al. 2002).

Infective organisms

Persistence of infective organism has been suggested to play a major role in the pathogenesis of CD. The IBD research group from the Royal Free Hospital have suggested that CD is a granulomatous vasculitis caused by the persistent presence of the measles virus, either from wild type infection or from a live-attenuated vaccine such as the Measles Mumps Rubella vaccine (MMR)(Wakefield and Montgomery 2000). Other research groups have challenged this theory. Even using sensitive PCR (polymerase chain reaction) detection techniques no independent group has been able to identify the measles virus in CD tissue. Epidemiological evidence has also failed to find a link with the vaccine (Feeney, Clegg et al. 1997, Table 2). In this study we compared the vaccination histories of 140 patients with IBD (83 with CD, 57 with UC) with those of 280 age and sex matched controls selected from the same GP lists. In this well-powered case-control study the rates of measles vaccination were very similar in those with CD, UC and subjects without IBD implying that vaccination was very unlikely to have been a major risk factor for the development of CD. The odds ratios for measles vaccination in CD in UC and in all IBD were all around unity suggesting no association between the vaccine and either type of IBD (table 2).

The other organism which has suggested as a possible cause of CD is the *Mycobacterium paratuberculosis*. This organism is capable of exciting a granulomatous reaction in cattle tissue, in the form of Johne's disease. In some cases *M. paratuberculosis* has been isolated from patients with CD (Engstrand 1995), however PCR studies suggest that the presence of this bacterium is not specific to CD (Hubbard and Surawicz 1999).

A case-control study of measles vaccination and inflammatory bowel disease.

Feeney M A, Clegg A, Winwood P, Snook J (1997).

Lancet 350(9080): 764-6.

Table 2 (a) Summary of patient details and

Odds ratios (95% confidence intervals) for IBD in vaccine recipients

	CD	UC	All IBD
Number of subjects	83	57	140
Median age at diagnosis	19 (15-22) *	20 (16-24)	19 (15-24)
Measles vaccine	1.08 (0.62-1.88)	0.84 (0.44-1.58)	0.97 (0.64- 1.47)

Table 2(b) Crude vaccination rates and age at first vaccination

	IBD group		Control group	
	Rate %	Age (months)	Rate %	Age (months)
Measles	56.4%	16	57.1%	17
Pertussis	70.7%	5	70.7%	6
Diph/Tetanus	92.9%	6	93.2%	6

This study had a 99% power to detect a 3-fold risk in CD in measles vaccine recipients (with a $p < 0.05$).

Dysregulated immune response to gut flora

Several forms of evidence suggest the role of gut bacteria in the pathogenesis of IBD. (a) The diversion of the faecal stream via a stoma can allow more distal inflammation to settle, especially in Crohn's disease (Winslet 1994). (b) IL-10 knockout mice only develop colitis when reared in non-sterile conditions (Kuhn, Lohler et al. 1993). (c) Titres of antibodies to *E.coli* are raised in patients with IBD (Tabaqchali, O'Donoghue et al. 1978). (d) Some strains of bacteria can alter the permeability of the gastrointestinal epithelium (Rocha, Laughlin et al. 2001). LPS (lipopolysaccharide), which forms bacterial cell walls is a potent stimulus to inflammatory cytokine production (Sartor 1995). The NoD2 gene variation which codes for a defective LPS receptor may prove to be a link between genetic and environmental factors in the causation of IBD (Hugot, Chamaillard et al. 2001; Ogura, Bonen et al. 2001).

1.3 Colorectal Carcinoma (CRC)

This is the second most common cause of death from cancer in the UK (after lung cancer). Incidence rises with age and the average age at diagnosis is between 60 and 65 years. There is no great difference in the rates of CRC between the sexes. Both genetic and environmental factors influence the risk of CRC. In figure 5 (p20) a section from a mucinous colorectal cancer is illustrated. This section has been prepared with the standard haematoxylin and eosin stain. The cells within the cancer show a variability of size and shape and are less organised than the cells in normal colonic epithelium.

1.3.1 Environmental factors and CRC

Immigrants from Japan which has a relatively low rate of CRC adopt an increased risk of CRC when they change to a Westernised lifestyle (Parkin, 1997). It has been suggested that diets high in fat and low in fibre may account for the increase in CRC in the Western world (Howe, 1992). Fat metabolism may promote the formation of carcinogens, which may have a longer time in contact with the mucosal surface due to slow transit times associated with low fibre high fat diets (Babbs 1990).

1.3.2 IBD and CRC risk

Longstanding colitis is a risk factor for CRC. The risk has been shown to be proportional to the duration of the colitis and is greater in those in whom the entire colon is affected (Eaden 2001). Other environmental risk factors include smoking and excess alcohol intake. Smoking generates oxidant stress and alcohol inhibits DNA repair.

1.3.3 Genetic factors and CRC

A family history of CRC at least doubles the risk of CRC. Certain families have larger numbers of individuals affected by CRC: and overall 15 % of siblings and 10% of offspring succumb to CRC. The risk of CRC in the general population is just under 6%. A variety of genetic defects have now been identified. However it is only a minority of all colorectal cancers that are associated with inherited defects, about 15 %. The other 85% are sporadic in individuals with no family history. The two most commonly recognised heritable cancer syndromes are familial adenomatous polyposis (FAP) and Hereditary Non Polyposis Colorectal Cancer (HNPCC). The *APC* gene functions as a gatekeeper and is mutated in >

85% of all CRCs (Powell SM 1992). In 10 -15% of cases mismatch repair genes are mutated. The *K-ras* gene is defective in 50 % of large adenomas and CRCs. Late in the process of carcinogenesis the p53 gene is often mutated, this being more often seen in malignant lesions (Grady 2000).

FAP

FAP results from a germline defect in the *APC* gene, which is inherited in an autosomal dominant fashion. This causes the development of diffuse colorectal polyps, usually visible by the second decade. Prophylactic colectomy is recommended as without it CRC is almost inevitable, usually before the age of 45 years (Jang 1997).

HNPCC(Hereditary Non Polyposis Colorectal Cancer)

This syndrome is also autosomal dominant in inheritance. It is diagnosed by clinical criteria (Amsterdam Criteria) (Lynch 1993): 3 successive generations affected, at least one first degree relative and one of these being less than 50 at the age of diagnosis. It is associated with cancers elsewhere: ovary, uterus, stomach, urinary tract and bile duct. Although recognised and diagnosed by clinical criteria recently mutations in 5 mismatch repair genes have been identified (Chung 1995).

1.3.4 Natural history and presentation of CRC

Most CRC are thought to develop through a sequence of changes in normal mucosa. First to form an adenoma this then develops into a CRC (Volgelstein 1988). This sequence of events is supported by the following observations: the location of polyps is very similar to that of CRC (two thirds in the left side of the

colon), resection of polyps reduces CRC risk, and benign adenomatous tissue is sometimes found very close to early CRC. In normal individuals the progression from normal mucosa to CRC is thought to take between 5 and 10 years. Therefore it may be possible to intervene to interrupt the CRC development by removing adenomatous polyps endoscopically.

Clinical features of colorectal cancer depend on the size and location of the tumour. Tumours of the caecum can remain relatively silent even when quite large and may present as iron deficiency anaemia or ill-defined abdominal pain. Cancers on the left side of the colon are more likely to present with bowel change either increased frequency, constipation, and change in calibre of the stool or blood mixed with the motion. Unfortunately these symptoms are non-specific and variable and there is a significant overlap of symptoms with benign conditions such as haemorrhoidal bleeding and irritable bowel syndrome. As described earlier it is important to achieve an early diagnosis as this greatly increases the likelihood of long-term survival.

1.3.5 Treatment of colorectal carcinoma

In localized disease a cure can be achieved by excision of the tumour and its draining lymph nodes. The outcome in rectal carcinoma can be improved by pre-surgical radiotherapy (Ajlouni 2001). In advanced CRC adjuvant chemotherapy with 5-fluorouracil and folinic acid agents has proven beneficial (Wolmark 1999).

1.4 The Oesophagus

1.4.1 Normal human oesophagus and gastro-oesophageal junction

The normal human oesophageal mucosa is made up of the muscularis mucosae and the lamina propria that are covered by stratified squamous cells. At the gastro-oesophageal junction there is an abrupt transition from the squamous epithelium of the oesophagus to the columnar type epithelium of the stomach. The mucosa of each region has a specialised function. The oesophageal stratified squamous epithelium has primarily a protective role, as the main function of the oesophagus is to transfer food from the mouth to the stomach by peristalsis. The mucosa of the stomach is glandular and is capable of secreting gastric juice (acid) and protective mucus. Normally the acidic stomach contents are retained in the stomach by the action of the lower oesophageal sphincter (LOS). The normal function of the lower oesophageal sphincter depends not only on the resting tone of the circular smooth muscle of the muscularis (internal LOS) but also the diaphragm (external LOS). The resting tone of the two muscular sheets that make up the diaphragm apply a pressure to the outer surface of the oesophagus. The diaphragm separates the thoracic from the abdominal cavity. In the normal situation the stomach lies below the diaphragm and at times of increased abdominal pressure, due to straining or inspiration, the diaphragm contracts so preventing reflux of the stomach contents in the stomach. As a wave of peristalsis delivers a bolus of food to the distal oesophagus there is a reflex co-ordinated relaxation of the LOS to allow the bolus to enter the stomach.

1.4.2 1.4.2 Gastro-oesophageal reflux disease (GORD)

Until relatively recently it was thought that acid reflux into the distal oesophagus occurred secondary to a faulty LOS. It has now been shown that in the majority of patients suffering from GORD the LOS functions normally most of the time. However, there are periods of LOS relaxation (not associated with a swallow), which allow acidic stomach contents to reflux into the distal oesophagus thus allowing the mucosal damage characteristic of GORD. These intermittent relaxations of the LOS are termed transient lower oesophageal sphincter relaxations (TLOSRS, Holloway 1995). During TLOSRS not only the internal sphincter of circular smooth muscle relaxes but also the external sphincter formed by the diaphragm, and this is thought to be mediated by a vagal reflex (Neuhuber 1987).

1.4.3 Barrett's oesophagus.

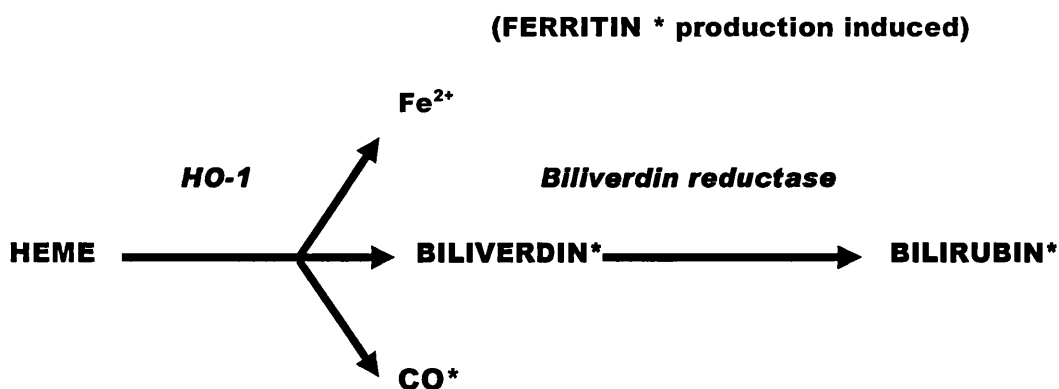
The term Barrett's oesophagus describes the replacement of the normal squamous epithelium of the distal oesophagus with columnar cells that demonstrate intestinal metaplasia. This change occurs as a consequence of GORD, exposure of the squamous epithelium to acidic conditions leading to a metaplastic change in the cells. Metaplasia is the process by which cells alter their morphological appearance in order to adapt to a change in their local environment. Thus a mature fully differentiated cell takes on the character of an entirely different cell type. Metaplasia can give rise to an increased risk of neoplasia. In the case of the metaplastic change of Barrett's oesophagus this is very important as it can then

progress to low grade and then high-grade dysplasia and ultimately adenocarcinoma of the oesophagus. Dysplasia is the histological term to describe changes in cell appearance, which occur when the rate of cell division increases and there is incomplete maturation of these cells. Dysplastic cells are unstable and are capable of becoming neoplastic i.e. developing into a cancer. Adenocarcinoma of the oesophagus is rising in prevalence in the Western world. Once cancer has developed, the prognosis despite all treatment is extremely poor. Even with best treatment five-year survival figures are in the region of 20-30 %. Microscopic metastases have often occurred at the time of clinical diagnosis; therefore even radical local resection with adjuvant chemotherapy or radiotherapy is unable to completely eradicate the cancer.

It would therefore be advantageous to intervene in the neoplastic process before cancer has developed. It is thought that adenocarcinoma of the oesophagus develops in areas of high-grade dysplasia, that in turn develop from low-grade dysplasia. The significance of Barrett's oesophagus is that this metaplastic process predisposes to the development of dysplasia and then adenocarcinoma, i.e. Barrett's oesophagus is a pre-cancerous condition. It is therefore desirable to intervene in the neoplastic process before cancer develops. Much research has been aimed at preventing the progression of normal healthy oesophageal tissue into Barrett's oesophagus in an attempt to interrupt the sequence of changes that ultimately lead to the development of oesophageal adenocarcinoma.

1.5 Background to HO-1 and reactive oxygen species

Heme-Oxygenase (HO) is the rate-limiting enzyme of heme metabolism, converting heme to free iron, carbon monoxide (CO) and biliverdin. Tenhunen first described its role in heme breakdown in 1968 (Tenhunen 1968).



* anti-oxidant

Heme forms the catalytic subunit of many proteins including the respiratory chain cytochromes (p450), cyclo-oxygenase (COX) and inducible nitric oxide synthase (NOS2) (Willis 1999; Guo, Shin et al. 2001). There are 3 isoforms of the HO enzyme, namely HO-1, HO-2 and HO-3. Each isoform is structurally distinct and encoded by different genes. HO-1 has a molecular weight of 32 kDa and is identical to the previously described Heat Shock Protein 32. HO-2 has a molecular weight of 36 kDa. HO-2 and HO-3 are constitutively expressed whilst HO-1 is the inducible form. HO-2 is expressed at fairly constant levels in cells throughout the body. HO-1 is expressed most abundantly in the spleen, the site of red blood cell degradation.

HO-1 can be induced by a number of agents or conditions: heme, UVA radiation, heavy metals, ischaemia, oxidative stress or cytokines. Cytokines capable of

HO-1 induction include: IL-1, IL-6, TNF α , and transforming growth factor (TGF β). HO-1 has a complex inter-relationship with nitric oxide synthase. For example, HO-1 can be activated by nitric oxide, and it can in turn deactivate NOS2 by degrading heme (the catalytic subunit of NOS2) (Tercanu V, Dhouib M et al 1998). HO-1 has been identified in many cell types in humans and other mammals. It has been suggested that its primary role is unlikely to be heme metabolism in all tissues but rather that this inducible form of the enzyme may have a more general defensive role by reducing damage caused by sustained oxidative stress (Applegate, Luscher et al. 1991).

The fundamental role of HO-1 is illustrated by a human case-report of HO-1 deficiency which resulted in growth failure, anaemia, abnormal tissue iron deposition, an increased sensitivity to oxidant stress and ultimately premature death (Yachie and Koizumi 2001). A mouse model of HO-1 deficiency showed that most animals died in utero and the survivors suffered similar problems to those described above in the human case-report (Poss and Tonegawa 1997). HO-1 can be induced by two groups of agents: firstly those which produce oxygen free radicals (e.g. hydrogen peroxide, UVA and menadione) and secondly by substances which reduce the amount of cellular glutathione (e.g. sodium arsenite, cadmium chloride and glutathione synthesis inhibitors) (Lautier, et al. 1992).

1.5.1 Heme, structure and function:

Heme acts as a prosthetic group binding tightly to some proteins and thereby altering their conformation and facilitating further ligand binding. Heme consists of an iron ion held within a cage formed by four pyrrole rings. Haemoglobin is

formed by four subunits each containing a heme prosthetic group. The presence of these heme prosthetic groups is essential to allow the binding of oxygen. In the respiratory chain cytochromes the heme group binds electrons. Heme is metabolised by heme-oxygenase to equi-molar amounts of biliverdin, CO and free iron (Fe^{2+}) (the reaction intermediates are illustrated in Appendix I). In mammals biliverdin is then further metabolised to bilirubin and excreted in bile, the iron being recycled in the synthesis of further heme.

Bilirubin is much less soluble in aqueous solution than biliverdin and requires conjugation in the liver prior to secretion in bile. It would appear a somewhat inefficient metabolic step to convert the readily soluble biliverdin to the relatively insoluble bilirubin; however formation of bilirubin may have other beneficial properties. Although biliverdin has some anti-oxidant activity this is greatly increased when converted to bilirubin. It has therefore been suggested that this degradative pathway has evolved to maximise an anti-oxidant influence.

(Stryer 1988)

In a recent population based study from the United States of America (over 176 million subjects) a significant association was found between low serum bilirubin levels and an increased risk of GI cancer. More specifically a rise in bilirubin of 1.0 mg/dl was associated with a risk reduction for colorectal cancer equivalent to an odds ratio of 0.257 (95% CI 0.254 - 0.260)(Zucker, 2004). The level of bilirubin was also shown to be significantly lower in smokers. A cancer protecting advantage associated with higher serum levels of bilirubin may account for the prevalence of genetic mutations such as Gilbert's syndrome in which a defective

form of the enzyme bilirubin glucuronase results in high levels of unconjugated bilirubin.

1.5.2 HO-1 has been shown to have a protective influence in other systems

As already mentioned, HO-1 metabolises heme (which is toxic) to produce biliverdin (which acts as an anti-oxidant), carbon monoxide (CO) and free iron. There are several lines of evidence to suggest that HO-1 mediates protective effects on physiological responses. Firstly, HO-1 has already been shown to be active in other systems. Much of the work has been conducted in the pulmonary and cardiovascular systems. Of all the products of HO-1, CO is probably the most potent anti-oxidant.

1.5.2.1 Pulmonary HO-1

In a rat model of acute pleurisy the activity of HO-1 rose (especially in mononuclear cells) as the inflammation progressed and this appeared to correlate with the onset of a resolution of the inflammatory response (Willis, Moore et al. 1996). In this model the addition of an HO-1 inhibitor resulted in increased inflammation and conversely the addition of an HO-1 inducer reduced overall inflammation. Exposure to very high concentrations of oxygen is a commonly used model of lung injury. The use of oxygen concentrations greater than 95% leads to the development of oxidant injury in a pattern similar to that seen in acute respiratory distress syndrome (ARDS). Rats exposed to conditions show induction of HO-1 in the pulmonary epithelial cells. (Lee, 1996). In an in vivo model rats transfected with an adenovirus capable of HO-1 production were resistant to hyperoxia, and survived longer than untreated controls. Some of this

protective effect may be mediated by the production of CO. Low levels of CO can render rats less vulnerable to hyperoxic injury (Otterbein, 1999). However there may be a threshold effect, very high levels of HO-1 and CO may be detrimental (Suttner, 1999). The protective effect of CO may be mediated by the down-regulation of pro-inflammatory cytokines such as $\text{TNF}\alpha$ and $\text{IL1}\beta$ and up-regulation of IL10 (Otterbein, 2000).

In acute asthma attacks the levels of exhaled CO are increased, suggesting increased HO activity induced as a response to acute inflammation (Zayasu, 1997). Smokers have also been shown to have higher levels of HO-1 expression in the pulmonary epithelium (Maestrelli, 2001). There is also a suggestion that smokers who develop emphysema are more likely to have an abnormal HO-1 gene promoter region, perhaps accounting for their susceptibility to develop chronic tissue damage in the context of long-term exposure to cigarette smoke (Yamada, 2000). A similar defect could affect the chronic inflammation of colitis and could perhaps account for the increased risk of cancer seen in total colitis.

1.5.2.2 Cardiovascular HO-1 research

Mouse heart transplant survival (with immuno-suppression) depends on HO-1. Rejection occurs if HO-1 is inhibited or if the recipient mouse is deficient in HO-1 (Soares, 1998). In the mouse model without the ability to express HO-1 long-term survival can be achieved by treatment with low dose CO, again supporting the concept that the cytoprotective action of HO-1 is primarily by the action of CO (Sato, 2001).

1.5.2.3 The inflammatory response of macrophages can be influenced by HO-1

Macrophages produce an inflammatory response when exposed to bacterial lipopolysaccharide (LPS). This response can be modulated by low dose CO in two ways. Normally in response to LPS macrophages produce a large amount of the pro-inflammatory cytokine TNF α (Beutler, 1985) and a small amount of the anti-inflammatory cytokine IL10. However if the macrophages are allowed to over express HO-1 or are pre-treated with low dose CO prior to LPS exposure then the balance is changed such that the production of TNF α is greatly reduced and that of IL10 greatly increased (Minamino, 2001). This may be directly relevant to the pathogenesis and disease activity in IBD, a process that is dependent on an aberrant inflammatory response driven in part at least by exposure to bacteria.

1.5.2.4 Animal models show the protective role of HO-1

In a rabbit model of contact lens related inflammation, increased HO-1 activity and HO-1 mRNA were found to correlate with reduced oedema (Laniado-Schwartzman, 1997). HO-1 is increased in rat models of cerebral ischaemia and less cell damage occurred after ischaemic injury in a transgenic mouse capable of overproduction of HO-1 (Panahian, 1999). Similar effects have been demonstrated in rat liver models, where stimulation of HO-1 can protect against ischaemic injury (Amersi, 1999). Finally, oxidant injury is a potent stimulus for apoptosis. It has been shown that fibroblasts over-expressing HO-1 are relatively resistant to oxidant stress and resulting apoptosis (Ferris, 1999).

1.5.3 Reactive oxygen species and chronic inflammation

Over the last two decades there has been increasing interest in the role of reactive oxygen species (ROS) in a wide range of chronic inflammatory conditions such as rheumatoid arthritis, pancreatitis and atherosclerosis. Cells living in aerobic conditions produce these ROS continuously. ROS have the capacity to cause tissue damage and a number of defensive mechanisms and enzyme systems have evolved to counteract and limit their effects. Various cells, notably neutrophils, use controlled production of ROS as part of the inflammatory response. ROS are responsible for tissue damage in most inflammatory conditions and control of their production and elimination is an attractive therapeutic target.

1.5.3.1 ROS definition and production

Free radicals are species with one or more unpaired electrons. Unpaired electrons are inherently less stable than pairs of orbiting electrons and so free radicals are unstable and highly reactive. Free radicals can damage carbohydrates, lipids, proteins and DNA. Free radicals are implicated in the production of ROS that are present in all cells and it is therefore essential that the production of such free radicals be under tight control. The combination of a radical and a non-radical will produce another radical, thus starting a chain reaction of radical production. Such a chain can be broken by a radical combining with another radical or by the action of anti-oxidant molecule such as vitamin E, vitamin C or anti-oxidant enzymes, for example, superoxide dismutase, glutathione peroxidase, glutathione reductase or catalase. HO-1 may also act as an important anti-oxidant enzyme in the

gastrointestinal tract. The broader term, reactive oxygen species, incorporates free radicals (with unpaired electrons) and oxidants such as hypochlorite and hydrogen peroxide. These oxidants readily participate in reactions to produce free radicals such as the hydroxyl radical (OH^\cdot). Transition metals such as iron play an important role in producing free radicals via the Fenton reaction: (Simmonds and Rampton 1993)



In the course of this reaction ferrous iron (Fe^{2+}) is converted to ferric iron (Fe^{3+}).

1.5.3.2 The role of iron in the production of the hydroxyl radical.

Transition metals such as iron (Fe^{2+}) promote the production of hydroxyl free radicals via the Fenton reaction. As the hydroxyl radical has such damaging potential, homeostatic mechanisms have developed to ensure that free transition metals are mopped up to prevent uncontrolled ROS production. Studies of iron chelation using desferrioxamine in pouchitis have yielded encouraging results, (but only published in abstract form) (Forbes, A A clinician's Guide to IBD 2001)

HO metabolises heme to biliverdin, CO and free iron. Biliverdin and its metabolite bilirubin are both anti-oxidant (Stocker 1987, Foretsi 2001) and low dose CO has anti-inflammatory actions. Iron is potentially pro-oxidant by facilitating the Fenton reaction and the production of the hydroxyl radical; however the release of iron also stimulates the synthesis of ferritin (Ferris 1999). Free iron (Fe^{2+}) binds to the apoprotein to form ferritin. By this mechanism iron is sequestered thereby limiting its availability for the Fenton reaction. HO can directly stimulate the production of ferritin that itself has anti-oxidant properties.

1.5.3.3 ROS and the pathogenesis of inflammatory bowel disease

The role of ROS in the pathogenesis of IBD has been well described by Rampton (Simmonds and Rampton 1993; Millar, Rampton et al. 1996). Accidental exposure of the colonic mucosa to hydrogen peroxide (previously used as a cleaning fluid) can give rise to an acute inflammatory response very similar in appearance to ulcerative colitis (Bilotta and Waye 1989). In the gastrointestinal tract free radicals are increased in areas of inflammation (Keshavarzian, Morgan et al. 1990). The presence of ROS have been demonstrated using chemiluminescence methods in the mucosa of patients with CD and UC, and the levels of ROS correlate with disease activity (Simmonds, Allen et al. 1992). (The chemiluminescence assay reagents luminol and lucigenin, in the presence of ROS have their electrons raised to a higher energy level and when these electrons revert to their normal energy levels they release energy in the form of photons, which can be quantified using a scintillation counter). ROS are also produced by activated polymorphonuclear leukocytes (Repine, Eaton et al. 1979), an increase in these cells being a cardinal feature of active inflammatory bowel disease. Production of ROS is increased at times of ischaemic reperfusion (Grisham and Granger 1988) probably as a result of the induction of xanthine oxidase (which produces superoxide). Several theories of the pathogenesis of both types of IBD (Crohn's and UC) invoke a model of ischaemic injury. Crohn's disease runs a more aggressive course in smokers, suggesting that micro vascular injury may play a role. This is also supported by the observation that heparin has been shown to be of some therapeutic value in the treatment of UC (Gaffney, Doyle et al. 1995). It has also been suggested that the colonic mucosa may be more vulnerable

to damage by ROS as the colon is relatively deficient in anti-oxidant defences. (McKenzie, Baker et al. 1996). Thus it may be that the tissue damage seen in IBD is as a result of the host anti-oxidant defences being overwhelmed by oxidant stress.

1.5.3.4 IBD drug therapy and anti-oxidants

The action of drugs commonly used to treat IBD may be in part explained by their influence on oxidant injury; the 5-aminosalicylate (5-ASA) agents have been shown to have a scavenger effect on superoxide radical formation by chelating free iron. (Gionchetti, Guarnieri et al. 1991). *In vitro* experiments have confirmed that these actions are achieved at 5-ASA concentrations similar to those found in the plasma of patients using oral 5-ASA preparations (Christensen, Fallingborg et al. 1990). Much higher concentrations of 5-ASAs are required to inhibit cyclooxygenase and lipoxygenase, suggesting that the therapeutic influence of this group of drugs may not involve these enzymes. Glucocorticoids inhibit the function of neutrophils and so may reduce ROS production (Baltch, Hammer et al. 1986). Several studies have investigated the possible therapeutic action of anti-oxidant agents in IBD. In a study of UC using sulphasalazine maintenance therapy the addition of DMSO as an oxygen scavenger or allopurinol (a xanthine oxidase inhibitor) resulted in better symptom control and longer periods of remission (Salim 1992).

1.5.3.5 Specific features of the action of HO-1 which may be relevant to IBD

A role for colonic epithelial cells in inflammatory and immune reactions is increasingly being recognized (Christ and Blumberg 1997). Ward's group and

others have demonstrated that colonic epithelial cells express pro-inflammatory enzymes such as COX-2 and NOS2 (Wright, Ward et al. 1997; Weaver, Russo et al. 2001) and also secrete members of the chemokine superfamily (IL-8, monocyte chemotactic factor-1 (MCP-1), monokine induced by interferon γ (MIG) and interferon inducible protein 10 (IP-10)) in response to the pro-inflammatory cytokines TNF α , IL-1 and IFN γ (Kolios, Robertson et al. 1996; Kolios, Wright et al. 1999; Dwinell, Luger et al. 2001). These cytokines in turn act as chemo-attractants for cells such as neutrophils, macrophages and T cells which are likely to contribute to the inflammatory response within the mucosa.

HO-1 offers an anti-inflammatory and anti-oxidant influence in many systems. If HO-1 is in some way reduced in activity this may lead to an imbalance of factors controlling the inflammatory response and in part account for the uncontrolled chronic inflammation that is the cardinal feature of IBD. A similar hypothesis has been put forward to account for the tissue damage of chronic pulmonary inflammation. Patients with emphysema have been found to have polymorphisms in the region of the HO-1 gene promoter, suggesting a possible mechanism for their susceptibility to increased tissue damage caused by smoking (Yamada, Yamaya et al. 2000). Could a similar decrease in HO-1 activity promote IBD? Even if HO-1 is found to be expressed in normal levels in IBD tissue it may still be possible to produce a beneficial anti-inflammatory effect by stimulating its activity within the gut to supra-normal levels.

At present, there is very little research investigating the role of HO-1 in the gut. However, HO-1 has been shown to be present in rat small intestinal epithelial cells (Rosenberg and Kappas 1989), and in the human colonic epithelial cell-lines, Caco-2 (Cable, Cable et al. 1993) and DLD-1. In the DLD-1 cell line, pre-treatment of cells with heavy metals (such as bismuth citrate) or NO donors, stimulated HO-1 and inhibited cytokine induced NOS2 activity (Cavicchi, Gibbs, Whittle. 2000). It is important to note that NOS2 is up-regulated in areas of active inflammation in IBD (Singer, Kawka et al. 1996). The gastro-protective actions of bismuth in peptic ulcer disease are well recognised; however the mechanism of this effect remains unclear. As bismuth stimulates HO-1 it is tempting to suggest that HO-1 may mediate this effect. Bismuth has also been shown to scavenge ROS in animal models of GI injury (Bagchi, 1999).

In a rat model of colitis induced by addition of trinitrobenzene sulphonic acid (TNBS colitis), HO-1 levels were increased in the colon after administration of the TNBS enema. When the colon was pre-treated with a HO-1 inhibitor tin mesoporphyrin the degree of inflammation caused by the TNBS was greatly increased, illustrating a protective action of HO-1 (Wang, Guo et al. 2001). It has recently been shown that low concentrations of CO, one of the products of HO-1 activity, can inhibit the production of lipopolysaccharide (LPS) induced pro-inflammatory cytokines/chemokines (TNF α , IL-1 β , MIP-1 β). These cytokines have been implicated in the inflammatory events leading to IBD (Otterbein, Bach et al. 2000). Thus CO (produced by HO-1) at low concentrations may be cytoprotective.

In other studies, vascular endothelial cells transfected with the HO-1 gene showed increased HO-1 mRNA. As a result increased HO-1 activity and reduced prostaglandin E2 production occurred. PGE2 is a product of COX-2 that is known to be up-regulated in IBD. Increased HO-1 activity was associated with a reduced COX-2 activity which returned to normal levels when HO-1 became normal (Haider, Olszanecki et al. 2002). For COX-2 to be catalytically active it requires a heme group, therefore reduced cellular levels of heme following metabolism by HO-1 lead to reduced COX-2 activity. The regulation of COX-2 has already been the target of drug treatment in gastro-intestinal inflammation and Barrett's oesophagus. (Buttar, Wang et al. 2002).

1.5.3.6 HO-1 may mediate the effect of IL-10 in IBD

Knockout mice deficient in IL-10 develop a colitis-like condition when reared in a non-sterile environment (Kuhn, Lohler et al. 1993). This illustrates the importance of bacteria in the pathogenesis of IBD and also suggests that IL-10 may offer a protective influence from this type of chronic inflammation in the gut. However, early clinical trials of intravenous IL-10 in Crohn's disease have been somewhat disappointing, showing only marginal clinical improvement, but no significant side effects (Fedorak, Gangl et al. 2000). Nevertheless, pre-treatment with IL-10 can protect mice from the development of experimental colitis (Barbara, Xing et al. 2000). Other studies have revealed that CO can also increase LPS-induced expression of the anti-inflammatory cytokine IL-10 (Otterbein, Bach et al. 2000). In turn, IL-10 can inhibit LPS-induced production of TNF- α , and induction of septic shock (Lee and Chau 2002). Inhibition of HO-1 protein synthesis or activity (using zinc protoporphyrin) significantly reversed the

inhibitory effect of IL-10. This observation suggests that HO-1 may act as a significant downstream effector of IL-10 (Lee and Chau 2002). IL-10 induced HO-1 via a p38 mitogen-activated protein kinase (MAPK)-dependent pathway. IL-10 treatment resulted in rapid phosphorylation of p38 (within 5 minutes), but had no effect on the other MAP kinases ERK or JNK. The inhibitory influence of IL-10 on LPS-induced inflammation can be blocked by cyclohexamide, suggesting that it involves a newly synthesized protein (Wang, Wu et al. 1994) which might be heme-oxygenase.

1.5.3.7 Relationship between NOS2 and HO-1

NOS2 is the inducible isoform of nitric oxide synthase. There are many similarities between the NOS2 and HO-1. Both enzymes result in the production of gaseous molecules capable of acting as secondary messengers. NOS2 produces NO and HO-1 produces CO. One notable difference in the products is that NO has a free electron and acts as highly reactive free radical, whereas CO is relatively inert. Many actions of NO have been found since its characterisation in the late 1980s as endothelium derived relaxing factor. It has a high affinity for heme groups and when bound to soluble guanylyl cyclase results in a four hundred fold increase in activity in this enzyme. CO also binds to guanylyl cyclase but only increases its activity by a factor of four. Both NOS2 and HO-1 can be activated by similar factors: cytokines and oxidant stress.

The action of NO within the gut still remains unclear, with both toxic and protective effects having been shown. NOS2 is increased in IBD (Boughton-Smith, Evans et al. 1993; Rachmilewitz, Stamler et al. 1995). Inhibition of NOS2 can reduce the severity of experimental colitis (Rachmilewitz 1995). Increased

expression of NOS2 leads to increase in NO production, superoxide and ultimately peroxynitrite, which cause tissue damage. NOS2 knockout mice are deficient in mucosal NO and develop colitis, underlining the mixed effects of NO in mucosal inflammation. Work in the DLD gut cell line showed that HO-1 stimulation can lead to an inhibition of cytokine induced NOS2 activity (Cavicchi, Gibbs, Whittle 2000). Similarly chemical inhibition of HO-1 has been shown to result in increased production of NO, both in an in vitro model of muscle strips (Chakder 1996) and in macrophages (Turcanu 1998). This down regulatory activity of HO-1 on NOS2 may be mediated by CO (a product of HO-1) binding to NOS2 via its heme group. CO has been shown to inhibit the action of NOS2. (White 1992). NO generally up-regulates HO-1 and increases CO production.

1.5.3.8 Recent developments in HO-1 in the Gut

Several studies have confirmed that the induction of HO-1 prior to oxidant stress can reduce tissue damage in animal models of colitis (Berberat 2005, Attuwaybi 2004). The role of CO in gut motility has attracted considerable interest, and the beneficial effect of CO can be blocked by the addition of the HO-1 inhibitor tin protoporphyrin (Korolkiewicz 2004).

The Pittsburgh group have explored the use of the products of HO-1 to reduce tissue damage following small bowel transplants in a rat model. This major surgery leads to significant post-operative ileus and risk of septicaemic shock. Four groups of rats were studied: (a) controls without surgery, (b) un-operated controls exposed to CO for 24 hours, (c) rats who received a small bowel transplant, and finally (d) a group who received a small bowel transplant and were exposed to CO peri-operatively. Inhaled CO (250ppm for 25 hours) one hour pre

transplant and 24 hours post transplant reduced the production of pro-inflammatory cytokines (IL-1 β , IL-6, NOS2 and COX-2) and improved post operative gut motility. (Nakao, 2003). In a second study of a very similar design the Pittsburgh group investigated the role of biliverdin. Biliverdin administered within the peritoneum was shown to increase survival and improve contractility of the transplanted gut in the post-operative period. Further experiments confirmed that the biliverdin group had a decrease in mRNA expression of the inflammatory cytokines IL-6 and IL-1 β , a reduction in mRNA expression of NOS2, COX-2 and ICAM-1 (intercellular adhesion molecule) and a decrease in neutrophil infiltration into the muscularis of the jejunum (Nakao 2004). This pair of studies confirms that the 2 of the products of HO-1 can result in functionally significant improvement in a small bowel transplant model. In this model the benefits of CO and biliverdin are mediated at least in part by different mechanisms: CO improves blood flow while biliverdin does not and biliverdin reduces the expression of ICAM-1 and reduces the number of infiltrating neutrophils while CO does not. It appears that CO and Biliverdin may have additive or even synergistic cytoprotective actions in the gut. The application of these observations in human subjects is likely to follow.

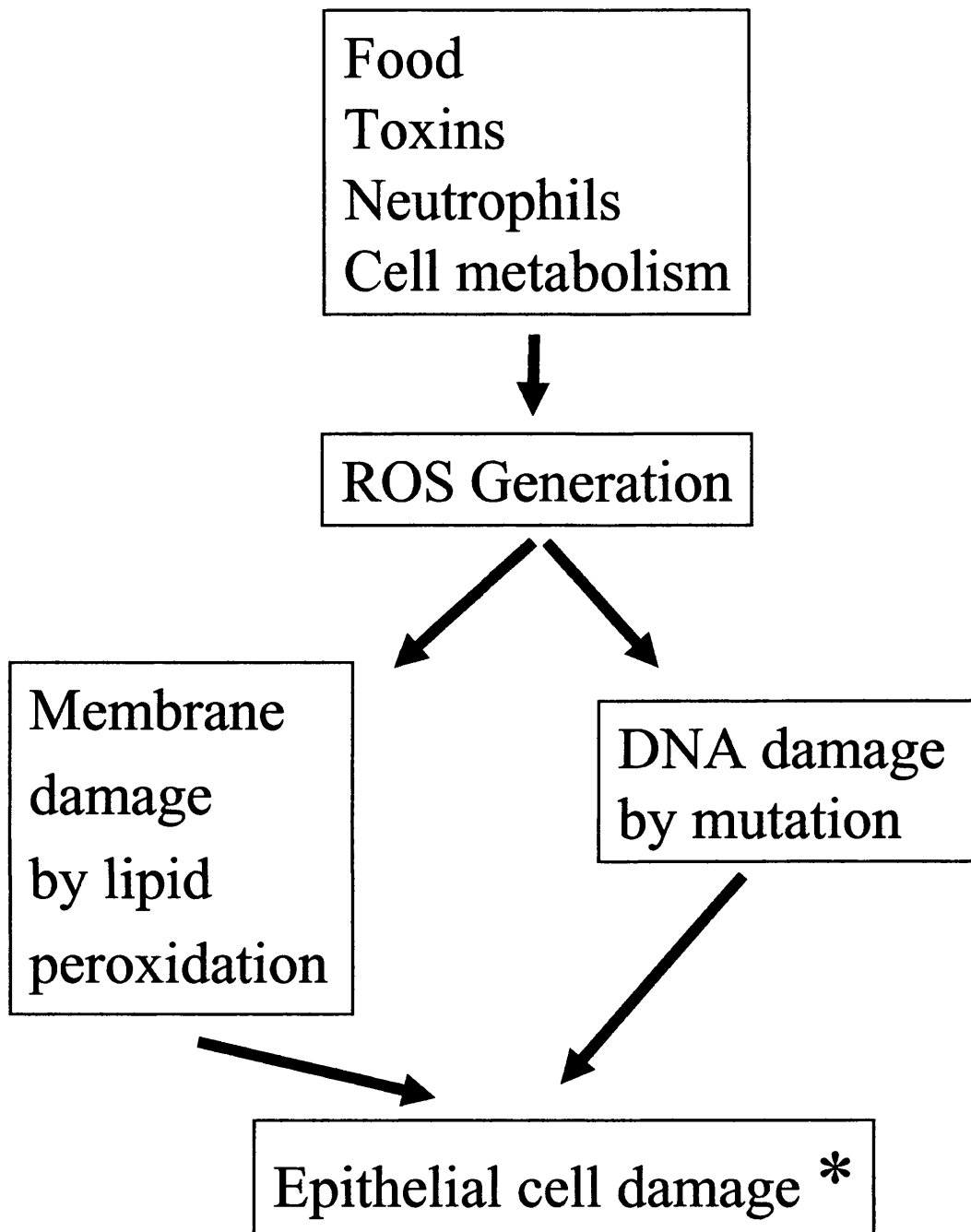
1.5.4 ROS and carcinogenesis

The development of cancer depends on multiple steps, involving somatic mutations and increased rate of cell growth. It has been suggested that the presence of ROS may stimulate some of the changes essential for carcinogenesis. For example, ROS can damage DNA leading to base changes and other mutations. ROS may also be responsible for activating potential carcinogens. Expression of

the *ras* oncogene protein leads to production of the superoxide anion and in turn to the production of oxygen containing free radicals such as hydroxyl, this may account for some of the carcinogenic influence of this oncogene. (Irani, 1997)

1.5.4.1 Link between chronic inflammation and carcinoma development

Chronic inflammation in the GI tract may lead to an accumulation of ROS by a number of possible mechanisms: (a) influx of neutrophils (Williams 1990), (b) increase in xanthine oxidase activity in epithelial cells (Parks, Bulkley et al. 1983), (c) increase in Fenton reactions (d) metabolism of arachonidate via the lipoxygenase pathway, and finally (e) the action of bacteria producing ROS. A number of cancers have been found to have relatively low levels of anti-oxidant enzymes such as superoxide dismutase and glutathione peroxidase ((Sato, Ito et al. 1992), hepatoma and (Jaruga, Zastawny et al. 1994), lung cancer)). When the anti-oxidant defences of the cell are overwhelmed then damage will result. Thus a loss of anti-oxidant influence may result in increased ROS production that leads to DNA damage directly and indirectly via lipid peroxidation (Figure 6, adapted from Farhardi 2002). Lipid peroxidation results in the destruction of cellular membranes, releasing potentially damaging lysosomal enzymes that can then cause further damage to the DNA. Protein oxidation may inactivate homeostatic enzymes within the cell cytoplasm. Unsaturated fatty acids when combined with hydroxyl radicals undergo lipid peroxidation. This may explain the link between diets high in unsaturated fats and an increased cancer risk (Bartoli, Palozza et al. 1993). This has been modelled in rats where an increase in the malignant transformation of colorectal adenomas has been demonstrated in animals fed on a diet high in unsaturated fats (Nicholson, Neoptolemos et al. 1991).



GORD → Oesophagitis* → BO → Adenocarcinoma

IBD → Chronic inflammation* → Colorectal Carcinoma

Figure 6 Suggested role of ROS in the pathogenesis of GI epithelial damage
(Farhardi et al 2002)

1.5.4.2 ROS and Colon Cancer

The link between ROS within a Western diet and increased colorectal carcinoma was suggested by Babbs (Babbs, 1990). He suggested that excess free radical production in stool could account for the increased incidence of colorectal cancer in the Western world. Intra-colonic free radical production is possible because the temperature conditions (stool temperatures between 30 – 60°C) favour superoxide production by bacteria. This then drives the Fenton reaction to produce the hydroxyl free radical. Aerobic bacteria in the periphery of the stool get sufficient oxygen from the mucosal surface of the colon to produce superoxide and hydrogen peroxide. Bile pigments act as iron chelators thereby facilitating the Fenton reaction and efficient hydroxyl production. The hydroxyl radical may then directly cause local damage or lead to the oxidation of pro-carcinogens. Conditions within the stool are mainly anaerobic and therefore most iron is reduced to the ferrous state (Fe^{2+}). This also favours the production of free radicals by the Fenton reaction:



Thus ferrous iron (Fe^{2+}) is converted to the ferric state (Fe^{3+}) and hydrogen peroxide to the hydroxyl radical.



Again the pattern of high levels of colorectal cancer in the affluent areas in the world would support the theory promoted by Babbs. Colorectal carcinoma rates are associated with diets rich in meat and consequently high levels of luminal iron. A high fat diet also contains more potential carcinogens. The Western diet, which has a high level of processed foods and fewer fresh vegetables, is low in fibre. A low fibre diet leads to an increased concentration of iron and free radicals

within the stool and decreased speed of gut transit increasing contact time of stool and stool generated ROS with the colonic mucosal surface. Inflammatory conditions such as chronic UC may increase the ROS production by the action of inflammatory cells within the mucosa, this could account for the increased risk of colorectal cancer in extensive colitis of long duration.

1.5.4.3 Increased risk of colorectal carcinoma associated with IBD

There is a well-documented increased risk of colorectal cancer associated with IBD affecting the colon; both for UC and CD this could be related to excessive ROS production. This risk increases with duration of disease and is most marked in those patients with total colitis. Cancer is not normally found before 8 -10 years of disease duration, and thereafter the risk is thought to increase by 0.5 -1 % per year. Cumulative risk over time is in the order of 2% at 10 years, 8 % at 20 years and 18 % at 30 years for all patients with UC (Eaden 2001). Best practice should be to offer screening colonoscopy to all patients with UC 8 – 10 years after first symptoms to assess the disease extent (Ridell 1990). For patients with total colitis surveillance, colonoscopy is suggested at 3 yearly intervals in the second decade of the disease, at 2 yearly intervals in the third and annually in the fourth and subsequent decades (Lashner 1988).

1.5.4.4 Chronic inflammation and increased risk of adenocarcinoma

Both chronic colitis and Barrett's oesophagus predispose to the development of adenocarcinoma. Both conditions occur in the setting of chronic inflammation in glandular epithelium. The ongoing oxidant stress in these conditions could account for the link between chronic inflammation and cancer. These conditions favour the generation of free radicals capable of damaging DNA and thereby

generating mutated cancerous cells Figure 1. It is therefore of interest to study the expression of anti-oxidant enzymes such as HO in normal and disease states to see if a deficiency in HO-1 could be implicated in the development of cancer.

1.5.4.5 Reactive Oxygen Species and Barrett's Oesophagus

Gastro-oesophageal reflux is associated with the development of oesophagitis and Barrett's oesophagus. Acid is probably the main agent causing this injury but bile acids and pancreatic enzymes have also been implicated (Goldberg, 1969; Demeester, 1976). The exact mechanism of this injury is not known but may be related to increased ROS production. Studies employing luminal enhanced chemoluminescence have demonstrated increased ROS production associated with oesophagitis and Barrett's oesophagus. The severity of inflammation correlates with the levels of ROS (Wetscher, 1997). The source of the ROS in the oesophagus is thought to be partly the epithelial cells (which contain xanthine oxidase) and partly neutrophils in the lamina propria. Azide and catalase only partially inhibit ROS production implying that in the oesophagus ROS production is not solely driven by neutrophils (Olyae, 1995). In contrast in UC, the ROS production is reduced by more than 80% by the application of azide and catalase implying a predominantly neutrophil based process (Sedghi, 1993).

Interventional animal studies have shown that the superoxide dismutase which scavenges superoxide radicals can reduce the severity of reflux oesophagitis (Naya, 1997). It has been suggested that ROS play a role in the development of oesophageal carcinoma by mediating oxidative damage to DNA. Animal models

of carcinoma of the oesophagus have shown that rats develop carcinoma of the oesophagus after oesophago-duodenal anastomosis and iron supplementation. In one study 60% of rats under such a regime developed carcinoma of the oesophagus by 35 weeks post surgery (Chen, 2000). Sacrificed rats with oesophageal cancer showed evidence of oxidative damage to DNA (increased 8-hydroxy-2'-deoxyguanosine). It has therefore been suggested that iron overload and reflux may account for increased oesophageal carcinoma. This may account for the increase in incidence in oesophageal cancer in the Western world, in an increasing affluent population a diet rich in meat and high in calories leads to relative iron overload and obesity which promotes acid reflux. Smoking is a risk factor for the development of oesophageal carcinoma and some of this effect may be related to increased endogenous production of ROS in the lungs and blood vessels in smokers. Smoking may also reduce endogenous anti-oxidant defences (Zhang, 1997).

Barrett's oesophagus is thought to develop by metaplasia of an immature oesophageal stem cell leading to the development of glandular cells (intestinal metaplasia). Under the ongoing influence of ROS it has been suggested that these metaplastic cells undergo a series of mutations resulting in increasing dysplasia and ultimately to the development of invasive carcinoma.

COX-2 has been shown to be raised in Barrett's oesophagus and adenocarcinoma of the oesophagus (Shirvani 2000) and high levels of COX-2 appear to predict poor outcome (Buskens 2002). In an endothelial cell model, over-expression of HO-1 was associated with a reduction in COX-2 activity and PGE₂ production. If

HO-1 activity is reduced in Barrett's oesophagus this could result in increased COX-2 activity and as a consequence an increased risk of the development of adenocarcinoma.

This raises the possibility that reduced oesophageal HO-1 activity in the context of GORD might predispose to the development of Barrett's oesophagus and possibly adenocarcinoma of the oesophagus. If this is confirmed it may offer a therapeutic target, by stimulating HO-1 or by adding other anti-oxidant enzymes it may be possible to reduce the cancer risk.

1.6 Aims

1.6.1 Hypothesis: Loss of appropriate regulation of HO-1 may cause a reduction in HO-1 levels resulting in an increase in reactive oxygen species in the gastrointestinal tract leading to the development of uncontrolled inflammation. This reduction in HO-1 may play a role in the development of inflammatory bowel disease, colorectal cancer and Barrett's oesophagus.

1.6.2 To investigate the expression of HO-1 in gut mucosa.

The aim of the present study is to investigate the expression of HO-1 in various tissue specimens from the GI tract. A comparison of immuno-histochemical staining between healthy tissue and that from patients with IBD, colorectal cancer will be made. A similar comparison between normal oesophagus and Barrett's oesophagus will also be undertaken. The level of HO-1 expression will be scored on a 3-point scale to aid comparison of the different tissue groups. The level of HO-1 expression will be scored both in the epithelial layer and in the lamina propria.

1.6.3 To investigate the effect of pro-inflammatory cytokines on HO-1 expression.

This work will be conducted in the colonic epithelial cell line HT29. To validate the experimental technique the influence of cytokine stimulation will first be examined by measuring COX-2 from HT29 cells, under the influence of TNF α .

HO-1 protein production will be confirmed by Western blotting. The skin fibroblast cell line FEK and Jurkat cells will be used to generate HO-1 to act as positive control samples for subsequent experimental work. Western blotting for HO-1 protein production will assess the effects of TNF α on HT29 cells, to investigate whether HO-1 production could be cytokine mediated. The effect of stimulation of HT29 cells ultra violet light (UVA) will then be explored.

2 Materials and Methods

2.1 Methods for immuno-chemical staining

2.1.1 Development of immuno-histochemical staining for Heme Oxygenase

To visualize the presence of HO-1 and HO-2 within tissue specimens the technique of immunohistochemical staining was used. A primary antibody specific to the protein of interest was first applied to the tissue specimen. A secondary antibody, with a peroxidase enzyme attached, was then applied to bind to the primary antibody. The peroxidase caused a brown colour change by reacting with a substrate chromogen solution (Diaminobenzidine, DAB). Thus the antibody targeted colour change (brown stain) could be used to identify the location of the proteins of interest.

A commercial kit called ChemMate (K5001/produced by DAKO) was used to localise HO. Although the ChemMate kit was developed for use in an automated system, it can be adapted to process small batches of slides manually. This is a labelled streptavidin biotin system (LSAB) (Figure 7). This system relies on the strong affinity between streptavidin and biotin. The secondary antibody has biotin attached; this binds to streptavidin that is in turn attached to the horseradish peroxidase, thus the peroxidase links to the primary and secondary antibody complex. Streptavidin has 4 binding sites for biotin. Some or all can be occupied simultaneously so that each biotinylated antibody reacts with several peroxidase-conjugated streptavidin molecules thereby increasing sensitivity (See figure 7).

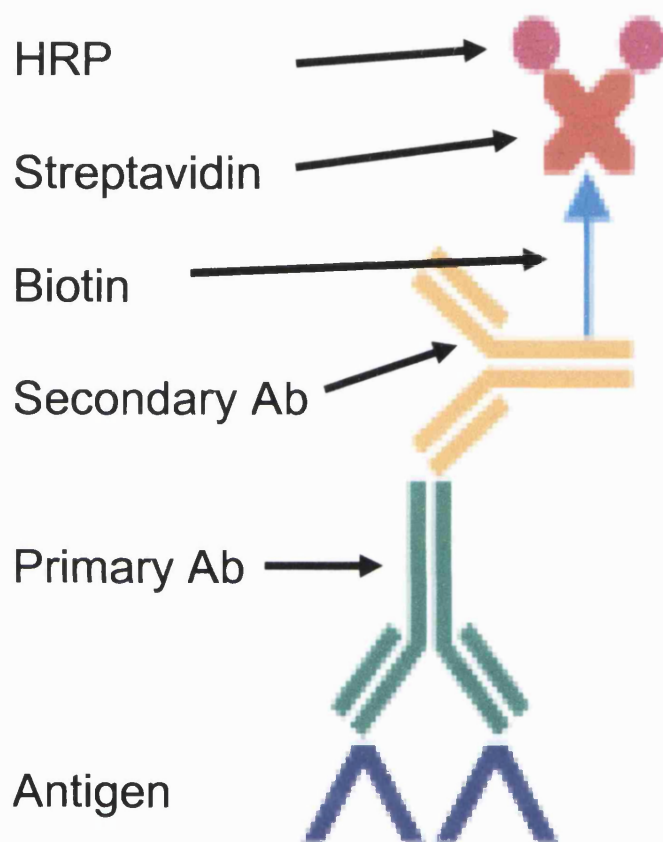


Figure 7 The labelled streptavidin biotin visualisation system (LSAB)
(HRP = Horseradish peroxidase)

2.1.2 Standard technique for immuno-histochemistry.

Tissue samples were either biopsies taken at the time of colonoscopy (by MF) or gastroscopy or colonic resection specimens. Consent for use of the resection specimens was gained from each patient. (See appendix II for patient information sheets). The resection specimens were used to allow examination of deeper structures of the bowel wall. Tissue was preserved in formalin and embedded in paraffin wax. Sections were cut to a thickness of 4 μm and mounted on slides. The slides were initially baked at 60°C for one hour to ensure adherence during subsequent processing. The slides were then treated to remove the paraffin wax: the slides were bathed in a solvent solution (citroclear) twice for periods of 4 minutes and then rinsed twice in industrial methylated spirit (for periods of 30 seconds). The slides were then thoroughly washed in tap water before the process of antigen retrieval.

The experiments by which the optimal conditions for HO-1 and HO-2 immunostaining were established are presented in the first Results chapter (3.1). Antigen retrieval was achieved by boiling the slides in a pressure cooker at full pressure for 2 minutes in a sodium citrate solution at pH 6.0. The slides were then rapidly cooled in cold water and after rinsing in TBS (pH 9.0) each slide was doused with a stream of TBS (pH 9.0) for about 10 seconds. During slide processing the slides were kept wet at all times. The slides were then blocked in BSA 5% in TBS (pH 9.0) for 1 hour to reduce non-specific background staining. After the blocking solution had been gently drained, the primary antibody was applied to each slide. The primary antibody solution was incubated overnight at 4°C. The optimal primary antibody concentration was found to be 1 in 500 for both HO-1

and HO-2. The HO-1 and HO-2 antibodies were both polyclonal rabbit antibodies bought from Santa Cruz. The primary antibody was diluted in DAKO diluent agent (S3022). The primary antibody was then washed off with a solution of TBS (pH 9.0) for 10 minutes. At this stage the secondary antibody was applied to each slide for 25 minutes at room temperature. The biotinylated secondary antibody solution was part of the ChemMate kit. Slides were then washed in TBS before applying the peroxidase blocking solution (agent S2023), 3 drops for 10 minutes. This solution contains hydrogen peroxide; the complexes formed between excess hydrogen peroxide and endogenous peroxidases are inactive. The slides were then washed in TBS once more followed by application of the horseradish peroxidase enzyme solution for 30 minutes. The slides were washed again in TBS and then the chromogen solution of diaminobenzidine applied for exactly 5 minutes. Finally the slides were washed in tap water and counterstained with haematoxylin for 90 seconds. The haematoxylin was first filtered to remove any crystals. The slides were then washed in water before briefly submerging in a solution of 70% industrial methylated spirit (IMS) and 0.1% hydrochloric acid. Finally to remove water from the slides they were submerged in a series of 3 containers of IMS then in 3 containers of xylene. The slides were then covered with pertex and coverslips.

2.1.3 Scoring immuno-staining

Slides were processed in mixed batches of healthy and diseased tissue, so that any minor variations in conditions would affect both healthy and diseased tissue equally. A simple negative control was always used, by omitting the primary antibody.

Scoring on a 3-point scale (Figure 8,9)

- 0 no stain
- 1 mild stain
- 2 strong stain

The intensity of staining was graded in the epithelium and the lamina propria separately. Two independent observers scored the slides. An agreement rate of 78% was achieved between the two observers (Dr M. Feeney and Dr N. Rooney). The score by Mark Feeney was used for statistical analysis.

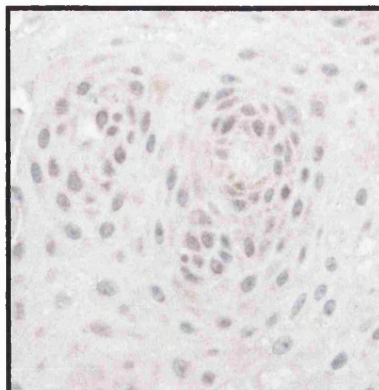
2.1.4 Final standardised conditions (Figure 10,11)

- Block for 1 hour at room temp in 5% BSA.
- Primary antibody at 1 in 500 overnight at 4°C.
- Secondary antibody (standard concentration as per ChemMate).
- TBS at pH 9.0 throughout.

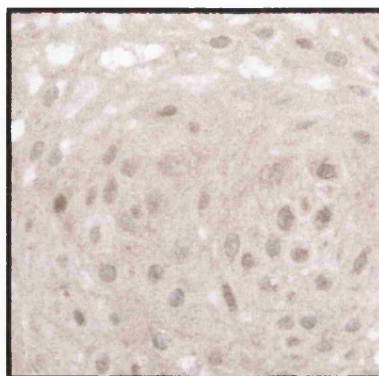
2.1.5 Antigen Retrieval

The slides were heat-treated in a pressure cooker at full pressure for 2 minutes. The standard slide preparation described above using formalin and paraffin wax leads to some loss of immuno-reactivity. It is thought that cross-linking of unrelated proteins within the specimen obscures some epitopes. To regain these lost epitopes a number of manoeuvres have been developed. Other groups have attempted to use proteolytic enzyme treatment, however in some cases this leads to the destruction of the epitopes. It has now become standard practice to heat-treat tissue prior to immuno-staining (MacIntyre, 2001). Use of a pressure cooker

(a) Score 0



(b) Score 1



(c) Score 2

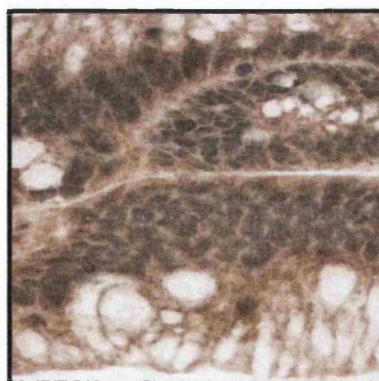
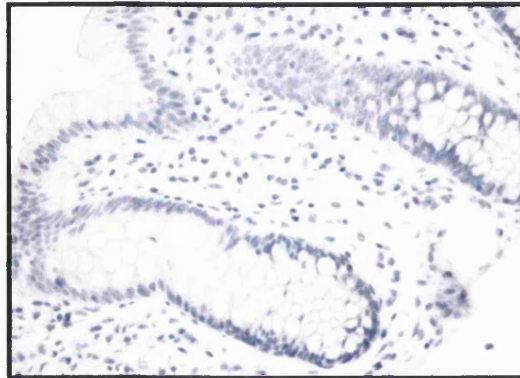
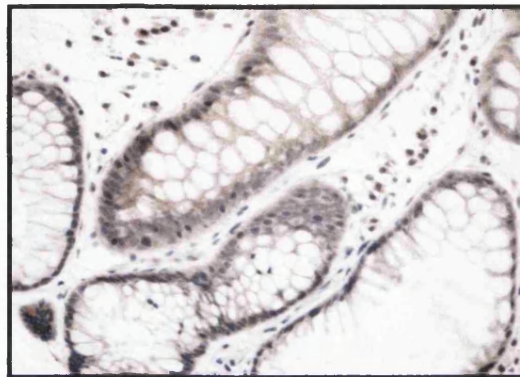


Figure 8 Slides to illustrate the 3 levels of staining intensity in oesophageal HO immuno-reactivity. (a) score 0= low, (b) score 1=medium and (c) score 2=high intensity. Brown stain indicates expression of the protein of interest. Original magnification 128 x. Full conditions described in Materials and Methods and summarised in Figure 10.

(a) Score 0



(b) Score 1



(c) Score 2

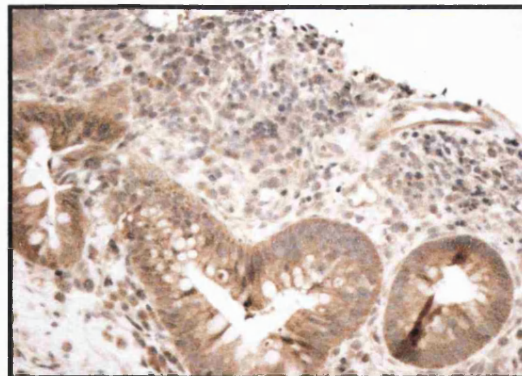


Figure 9 Slides to illustrate the 3 levels of staining intensity in colonic HO immuno-reactivity. (a) score 0= low, (b) score 1= medium and (c) score 2= high intensity. Brown stain indicates expression of the protein of interest. Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

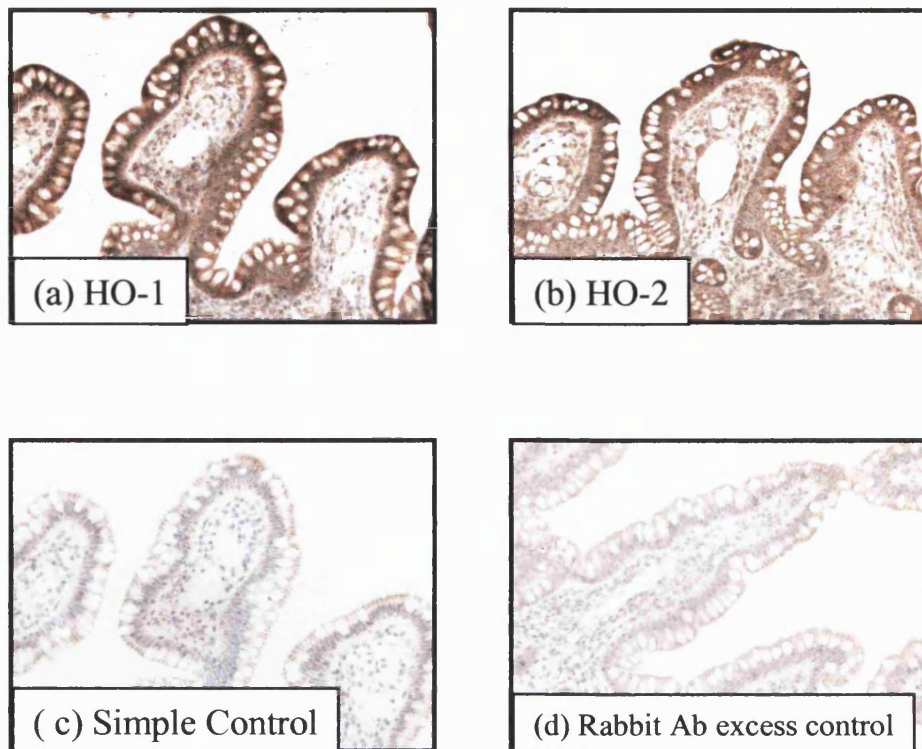


Figure 10 Standardized conditions for HO immuno-reactivity (illustrated in small bowel biopsies) After antigen retrieval the slides were treated in a blocking solution of 5% BSA in TBS (pH 9.0) for 1 hour. The primary antibody solution was incubated overnight at 4°C at a concentration of 1:500, the biotinylated secondary antibody secondary antibody was applied to each slide for 25 minutes at room temperature. Then the peroxidase blocking solution was applied, followed by the horseradish peroxidase enzyme solution for 30 minutes. After washing a chromogen solution of diaminobenzidine applied for exactly 5 minutes. Finally the slides were washed in tap water and counterstained with haematoxylin for 90 seconds. Small bowel biopsies were stained with primary rabbit polyclonal anti-human antibodies supplied by Santa Cruz: (a) HO-1 , (b) HO-2 (c) Control (absence of primary antibody), (d) Control with non-specific rabbit immunoglobulin. Brown stain identifies specific protein expression. Original magnification 128 x.

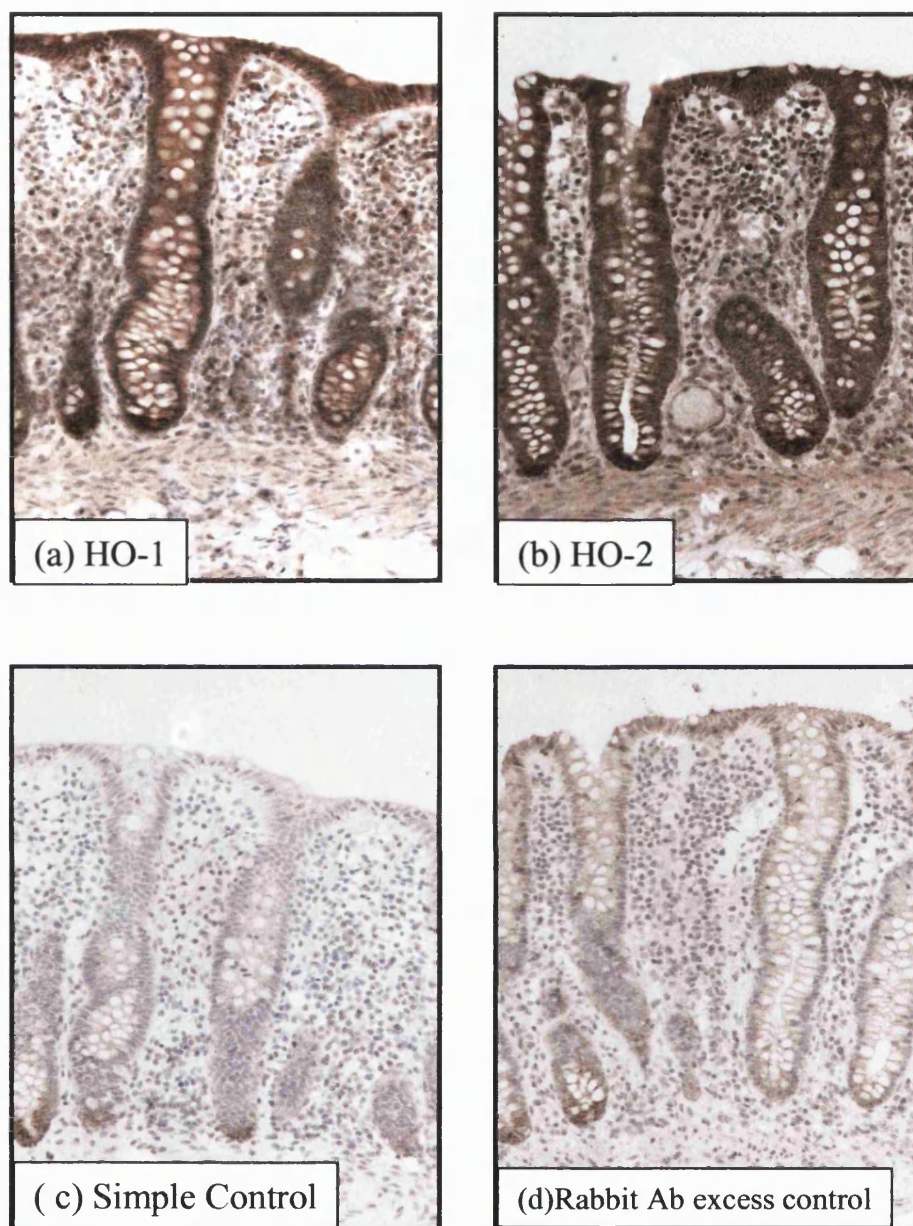


Figure 11 Standardized conditions for HO immuno-reactivity (Colonic). Colonic biopsy samples and deep resection specimens were stained with primary rabbit polyclonal anti-human HO-1 antibody (Santa Cruz) at a concentration of 1:500. (a) HO-1 , (b) HO-2 (c) Control (absence of primary antibody), (d) Control with non-specific rabbit immunoglobulin Brown stain identifies specific protein expression. Original magnification 128 x. Full conditions described in Materials and Methods and summarised in Figure 10

allows a brief intense period of heat treatment. The mechanism of action of antigen retrieval remains controversial. Heat is thought to reverse some of the cross-linking that has been induced by the formalin while leaving other cross-links intact so that the proteins are not denatured.

2.1.6 Antigen Excess / Pre-absorption controls

The best type of control for immuno-histochemical procedures is the antigen excess control. The lowest primary antibody concentration to give a consistently positive result is first established. Then the primary antibody is incubated with a blocking peptide specific to the protein of interest. The blocking peptide is a small highly specific part of the protein of interest, i.e. a section that differentiates it from other proteins. The blocking peptide is added in excess by a factor of ten by weight. Thus the entire available primary antibody is specifically bound and unavailable to bind to the tissue sample. This mixture is then used instead of the primary antibody in the protocol. As there is no unbound primary antibody available to bind with the protein of interest this should result in no colour change. Unfortunately there were no specific blocking peptides available for the Santa Cruz antibodies for HO-1 or HO-2. The possibility of using HO blocking peptides from other companies was explored; unfortunately none that specifically block the Santa Cruz antibody were available.

Table 3

Table 3	Santa Cruz Ab Base-pairs	Stressgen Ag Base-pairs
HO-1	184-288	Upstream from 261
HO-2	293-311	1-291

Table 3 illustrates the reason a pre-absorption control could not be performed for the Santa Cruz antibodies employed. The table shows that the antibody from Santa Cruz does not correspond to the blocking peptide that was available from Stressgen. As a more crude form of antigen excess control the primary antibody was incubated with non-specific rabbit immunoglobulin. These non-specific immunoglobulin controls are shown in Figures 10 and 11.

2.1.7 HO-1 and HO-2 Homology

In human HO proteins there is only 42% homology of the amino acid sequences. The regions with the greatest similarity are between 125-150 in HO-1 and 144-169 in HO-2 (Wilks 2002). This conserved region codes for the distal helix and is thought to be the fingerprint motif of the HO proteins. The Santa Cruz antibodies are raised against regions distant from this shared amino acid sequence (see table 3) thus allowing the antibodies to differentiate the two proteins. The Santa Cruz antibodies used for HO-1 and HO-2 were both polyclonal rabbit antibodies i.e. they react with a variety of epitopes on the target antigen. Polyclonal antibodies tend to have a range of low and high affinity against several epitopes; this reduces the loss of specific antibody binding during the washing stages of the immunostaining process.

2.1.8 Statistical Analysis

Non-parametric statistical tests were used to analyse the significance of the differences in intensity of the HO-1 immuno-reactivity. In this study intensity scores are ordinal data i.e. the intervals between scores do not represent a precise difference in quantity. Ordinal data can be placed in a meaningful rank order; however an intensity score of 2 does not represent intensity twice as great as that of a case that scored 1. When comparing two sets of data the Mann-Whitney 'U' test (MWU) (also called the Wilcoxon rank-sum test) was used. In the present work the MWU test was used to compare the intensity scores from the oesophageal biopsies, that is normal vs. Barrett's oesophagus.

The MWU test is used to test whether two independent groups are drawn from the same population. The null hypothesis is that the median values are the same. When three sets of data were compared the Kruskal-Wallis test (KW test) was used. The KW test was therefore used to compare the data sets from the colon biopsies; normal vs. IBD vs. colorectal cancer. When multiple sets of data are compared it becomes more likely that one of the results will reach significance at the $p=0.05$; 1 in 20 results will reach this level of significance by chance. Multivariate analysis of variance such as the KW test is designed to adjust for this. Neither the MWU nor the KW tests require the data to be normally distributed, by analysing by rank order they also avoid problems related to the undue influence of outlying values. Non-parametric tests are less powerful than the corresponding parametric test i.e. a non-parametric test is less likely to detect a real effect.

The null hypothesis holds that the scores from the two groups are very similar such that if all the data from the two sets were combined into one large set and placed in rank order then the data from both sets would be randomly/evenly scattered through the combined set. If the null hypothesis is rejected then when the combined set is arranged in rank order, data from one sample should concentrate to one end of the scale while data from the other set should be at the other end of the scale i.e. suggesting that the two data sets are significantly different.

To calculate the MWU statistic the individual data sets are first placed in rank order within their own data sets. Then the two data sets are ranked concurrently, in ascending order across the combined set. The MWU statistic is related to the sum of the ranks for each data set and the number of variables in each set. Similarly the KW statistic is based on the sum of the ranks for each sample set when all the sample sets have been ranked in one combined set concurrently.

Where two or more scores are the same, the ranks are adjusted for ties. That is to say that if the 9th and 10th scores in rank order of the combined set are identical then they each are assigned the score 9.5. If in a three group comparison three scores are identical e.g. 5th, 6th and 7th ranked scores are all the same then they are all ranked 6th, the next variable in this sequence will then of course be ranked 8th. If all the data in the two groups under comparison are identical then there will be no difference in the rank order.

In this study the MINITAB software was used to calculate the p value for the MWU statistic. In this case a p value of < 0.05 was taken as significant and such values suggested that the null hypothesis (that there was no difference between the two groups compared) could be rejected at the 95% confidence level. Where the p value was > 0.05 then the null hypothesis was accepted. In all the data presented the values were adjusted for ties. A two-tailed test was used, as a difference in intensity scoring either increased or decreased was important.

2.1.9 Immuno-staining reagents

Chem Mate Kit K5001

The primary antibody was used in 400 μ l of CHEM MATE diluent.

Na Citrate pH 6 to make upto 10 L

29.4g Na Citrate

Titrate HCl to achieve a pH of 6

TBS (Tris buffered saline) made upto 4 L

Tris	24.2 g
NaCL	34.0 g
HCl	To pH 9.0

2.2 Methods for Western blotting

2.2.1 Western Blotting

The Western blotting technique was used to detect the relative amounts of specific proteins. First the protein mixture was passed by electrophoresis through an SDS gel (sodium dodecyl sulphate), and then transferred onto a nitro-cellulose gel. The distance moved by each protein is determined by its molecular weight. The nitro-cellulose membrane was then treated with a primary antibody specific to the protein of interest. The membrane was then placed in a solution of a secondary antibody (tagged with horse-radish peroxidase) that would bind a specific primary antibody. Thus when the secondary antibody is visualised it identifies the relative amounts of the protein under investigation.

2.2.2 Assembly of a Miniprotean gel

Varying concentrations of the SDS gel were used to accommodate proteins of differing sizes, 7.5% SDS gel for COX and 10% for HO-1 (See Materials). The resolving gel was poured between two glass plates and allowed to set, taking approximately 20 minutes. To ensure a level border the gel was overlaid with MQ (Milli Q) water, and when the resolving layer had set the MQ was removed and the stacking layer poured on top. A plastic comb was then immediately placed into the liquid stacking gel to form a series of wells within the stacking gel as it set. When this had set the comb was removed to reveal the empty sample wells. These were then washed before use. On each gel the first well was loaded with molecular weight markers. 20 µl of sample were reloaded into each lane. Initially the gel was run at 80 V until the blue marker had reached the resolving gel, then the voltage was increased to 180 V until the gel had reached the bottom edge of

the resolving gel. The gel was then carefully dissembled and placed onto the nitro-cellulose membrane for transfer of the proteins from the gel to the nitrocellulose paper.

2.2.3 Membrane Transfer

Placing the gel on a nitro-cellulose paper and applying a voltage difference between 2 graphite electrodes across them achieved transfer of the separated proteins. Four dampened pieces of filter paper (cut to the same dimensions as the membrane (8.5 x 6 cm)) were stacked on the lower electrode, and onto these was placed the nitro-cellulose membrane and then the gel. The pile was then completed with 4 more pieces of the filter paper. As each layer was added care was taken to remove any air bubbles by gently smoothing the surface with a glass roller. The upper electrode was then placed on the top of the stack. To transfer the proteins a current of 43 mA per blot was used for 1 hour (0.8 mA/cm^2 per gel). After this the stack was dissembled and the membrane stained with Ponceau red to confirm that protein transfer had taken place with equal loading and also to facilitate the identification of the protein markers whose positions were then marked on the membrane with a pencil. The membrane orientation was indicated by cutting off a small corner from the top right hand corner of each gel. The Ponceau was then washed off. The primary antibody was applied, and then washed off before the secondary antibody was applied. The membrane was then ready for developing.

2.2.4 Developing Western Blot

The nitro-cellulose membrane was washed before being treated with Enhanced Chemiluminescent (ECL) reagent for 1 minute. The membrane was then wrapped

in transparent cooking film and placed in a lightproof film case. The membrane was exposed to X-ray film for a number of minutes and then developed in an RG11 Fuji X-ray film developer. The hard film was over-laid onto the original membrane so that the molecular weight markers could be recorded onto the film. Then the membrane was washed in TBS and dried in filter paper. It was stored at 4° C and later re-analysed for another protein to demonstrate equal loading of protein. To make meaningful comparisons of the relative amounts of the protein of interest in each lane it is essential to load equal amounts of total protein. The Bradford technique described above allows equal masses of total protein to be loaded into each lane, 30 µg in these experiments. Western blots were developed with primary antibodies directed against a protein whose concentration would be constant in cells independent of the experimental conditions. Thus a Western blot for such a protein should produce a consistent band at the same molecular weight and of equal intensity across all of the experimental samples, thus demonstrating equal loading.

2.2.5 Stripping Western Blot

In order to probe blots a second time for a different protein the blots were stripped by immersion for 30 minutes in a stripping buffer at 60°C in a sealed sandwich-box. Prior to this treatment with stripping buffer the blots were re-hydrated in TBS for 30 minutes. Following this the blots were rinsed in 5 lots of TBS each for 5 minutes. Once this was completed the blots could then be placed in a blocking solution and then exposed to primary and then secondary antibodies as described above. Blots were stripped and probed again for a second protein to assess equal loading. For COX-2, COX-1 was chosen to assess equal loading, as COX-1 is a

constitutive version of the enzyme and does not vary under stimulation. ERK was probed to assess equal loading in the case of HO-1.

2.2.6 Western blotting conditions Table 4

	Blocking	Primary Ab	Secondary Ab
COX 2	5% Marvel in TBS Tween 0.05% azide 2 hrs room temp	Santa Cruz Polyclonal Goat 1:500 1% Marvel Overnight at 4° C	Rabbit Anti-goat 1:10,000 2.5% Marvel 2 Hrs room temp
HO-1	5 % Marvel TBS 2 Hrs Room temp	Santa Cruz Polyclonal Rabbit 1:500 5 % Marvel Overnight at 4° C	Goat Anti-rabbit 1:2000 5 % Marvel 2 Hrs room temp
ERK	5 % Marvel TBS	Polyclonal Rabbit 1:1000 TBS Tween 3 Hrs room temp	Goat Anti-rabbit 1:10,000 TBS Tween 1 hr room temp

2.2.7 Materials for Western blotting

SDS-PAGE Buffers / Western Solutions

<u>4X Resolving Gel Buffer</u>	<u>g/500 ml</u>	
1.5 M Tris	90.86	pH 8.8
0.4% SDS	2.0	

<u>4 X Stacking Gel Buffer</u>	<u>g/500 ml</u>	
0.5 M Tris	30.29	pH 6.8
0.4% SDS	2.0	

<u>Reservoir Buffer</u>	<u>g/ 5 l</u>	
25 mM Tris	15.15	
192 mM Glycine	72	
0.1% SDS	5	

<u>Semi-Dry Transfer Buffer</u>	<u>g/l</u>	
48 mM Tris	5.81	
39 mM Glycine	2.93	
SDS	0.375	
20% (v/v) Methanol	200 ml	

<u>Washing Buffer (Tris buffered Saline, TBS) g/l (10x stock)</u>		
10 mM Tris	12.11	pH 7.5
100 mM NaCl	58.44	

<u>TBS Tween (0.1% Tween 20)</u>		
TBS x1	500 ml	
Tween 20	0.5 ml	

Table 5

	Resolving Gel		Stacking Gel
(range kDa)	7.5%	10%	
	(70-200)	(20-100)	
dH ₂ O	9.84 ml	8.17 ml	6.85 ml
resolving buffer	5.0 ml	5.0 ml	(Stack) 3.0 ml
Bis-Acryl(30%)	5.0 ml	6.67 ml	2.0 ml
10% APS	150µl	150 µl	150 µl
TEMED	15µl	15µl	10 µl

Lysis Buffer

This buffer was made fresh, stored on ice and used within 4 hours of production (to avoid enzyme degradation).

Per 10 ml of Buffer

50 mM Tris-HCl pH 7.5	0.5 ml of 1M
150 mM NaCl	0.3 ml of 5M
1% Nonidet P40	1 ml of 10% (v/v)
10% Glycerol	1 ml of 100% (v/v)
5 mM EDTA	0.1ml of 0.5 M pH 8.0
1 mM sodium vanadate	0.1 ml of 0.1M
1 mM sodium molybdate	0.1 ml of 0.1M
10 mM sodium fluoride	0.2 ml of 0.5M
40 µg/ml PMSF	5 µl of 80 mg/ml stock in DMSO
0.7µg/ml Pepstatin A	5 µl of 1.4 mg/ml stock in Methanol
10 µg/ml Aprotinin	5 µl of 20 mg/ml
10 µg/ml Leupeptin	5 µl of 20 mg/ml
10 µg/ml Soyabean Trypsin inhibitor	5 µl of 20 mg/ml
dH ₂ O	6.7ml

Blocking Solution

TBS Tween + 5% non-fat milk (w/v) (Marvel)

<u>5 x Sample Buffer</u>	<u>For 50 ml</u>
5% SDS	2.5 g
50% Glycerol	25 ml
200mM Tris –HCl ph 6.8	10 ml of 1M
MQ Water	14 ml
Bromophenol Blue	1 ml approx
(5% 2-ME add on the day of use 50 µl /1 ml)	

<u>Stripping Buffer</u>	<u>100 ml</u>
200 mM Tris / HCl pH 6.8	40 ml
10% SDS	20 ml
H ₂ O	40 ml
β Mercapto-ethanol	770 µl

Cell Culture Medium for HT29

McCoy's Medium 5A supplemented with	Penicillin	10 units/ml
	Streptomycin	10 mg/ml
	Fungizone	0.5 mg/ml

Medium for freezing HT29

- 10% (v/v) DMSO
- 40% (v/v) FBS
- 50% (v/v) Complete McCoy's Medium

2.3 Cell culture

2.3.1 HT29 Cell culture

The HT29 cell line was isolated originally from a primary colonic tumour in a 44-year-old Caucasian female. These cells retain many of the features of colonic epithelial cells: epithelial polarity, the presence of the actin-binding protein and enterocyte differentiation. There were some limitations to the use of HT29 cells for modelling colonic epithelium. Although this cell line does have a cell surface receptor for IL-10, it does not have a fully functioning intracellular portion and as a result this cell line does not respond to IL-10 signalling. This is of some importance, as IL-10 is known to have anti-inflammatory effects in IBD; (NB IL-10 knockout mice develop colitis when reared in non-sterile conditions). Interestingly, researchers in Professor Ward's group at Bath have shown that in HT29 cells, IL-4 and IL-13 inhibit COX2 and NOS2 production but IL-10 does not have the same effect.

The European Collection of Animal Cultures (ECACC) provided the HT29 cell line. Cells were cultured in 80 cm² flasks. Cells were fed on alternate days with Modified McCoy's medium supplemented with penicillin (10u/ml), streptomycin (10ug/ml) fungizone (0.5ug/ml) and 10% foetal bovine serum (FBS), (this combination was referred to as complete McCoy's Medium). HT29 cells were incubated in an atmosphere of 5% CO₂ at a temperature of 37°C. For Western blotting experiments HT29 cells were then cultured to 90% confluency prior to stimulation in 6 well plates. Cells in the main culture flask were initially washed with PBS (without Ca²⁺ and Mg²⁺) to remove the medium then a Trypsin-EDTA mixture was added and the cells incubated for 5 minutes. When the cells were

seen to have detached from the flask the action of the trypsin was inhibited by the addition of complete McCoy's medium. The suspension was then centrifuged for 5 minutes. The pellet was re-suspended in complete medium and the number and viability of the cells assessed using a Neubauer haematocytometer. A small sample of the cellular suspension was mixed with Trypan Blue and the proportion of dead cells (stained blue) could then be calculated. Cells were then seeded into the 80 cm² flask (for further cultures) or into 6 well plates for Western experiments. A variety of dishes were used and it was found that adequate protein yields could be achieved using 6 well plates (Nunc). In these plates 3.5x10⁶ HT29 cells grew, providing approximately 1.5mg of protein (measured by the Bradford method). Cells in flasks and 6 well plates usually took between 5 and 7 days to reach confluency.

2.3.2 Cell stimulation

Prior to stimulation the cells were starved overnight in serum free medium. On the morning of the stimulation the medium was again changed an hour before the addition of the active agents (cytokines or other stimuli were added). To arrest the reaction the cell plates were first washed twice with PBS and then 500µl of ice-cold lysis buffer was added to each sample before the cells were vigorously scraped off the culture plate. The cellular solution was then placed into a 1.5 ml specimen tube on ice for 5 minutes, rotated at 4°C for 15 minutes and finally centrifuged for 15 minutes. At the end of this process the cells had all undergone lysis, and the cellular debris had formed a pellet at the bottom of the specimen tube. At this point the sample was stored by freezing to -80°C. Prior to Western

blotting, protein concentration measurements were carried out by the Bradford technique.

2.3.3 Freezing / Thawing HT29 cells

HT29 cells were stored in a solution of 10% DMSO, 40% FBS and 50% McCoy's medium in liquid nitrogen. The cells were thawed by placing the vial in a warm water-bath for 5 minutes before transferring to a tube containing 10 ml of warmed complete McCoy's medium. To remove the DMSO this mixture was centrifuged for 5 minutes, the supernatant discarded and the pellet re-suspended in complete McCoy's medium prior to seeding in an 80 cm² flask. To freeze the cells, suspensions of cells were placed in cryotubes (Nunc) cooled gradually overnight and then transferred for long-term storage into liquid nitrogen tanks.

2.3.4 Protein concentration measurements by the Bradford Technique.

To make meaningful comparisons between different samples it is essential to load the same amount of total protein for each sample. To measure protein levels the amount of protein was estimated by comparing to standard dilutions of bovine serum albumin (BSA), typically 2, 5, 10, 20, and 40 µg/ml. Five µl volumes of the standard protein solutions and of the specimens under investigation were then added to 1 ml of Bradford reagent. 100µl volumes of these solutions were then loaded into a 96 well plate and then analysed using a Dynatech MR5000 plate-reader. Bradford reagent undergoes a characteristic colour change that is dependent on the concentration of the protein added. Thus the standard solutions

define a curve to which the experimental samples can be compared. Once the protein concentration of each sample was known it was possible to dilute each sample with differing volumes of lysis buffer so that they all ultimately had the same final total protein concentration. Sample buffer was then added and the samples boiled for 10 minutes. In this work a final concentration of 150 $\mu\text{g}/100\ \mu\text{l}$ was used so that when loading the Western gel with 20 μl of each sample these contained 30 μg of protein.

2.3.5 HO-1 Positive Control Preparation

FEK₄ cells are a skin fibroblast cell line. When stimulated by UVA radiation they produce HO-1 with a good yield of protein being achieved after 6 hours. FEK₄ cells were seeded and grown to 90% confluence on a 90 mm tissue culture plate in an incubator. The cells were then drained of their media, which was kept for later use during the incubation phase. The cells were then washed in PBS (without $\text{Ca}^{2+}/\text{Mg}^{2+}$) and placed in PBS with $\text{Ca}^{2+}/\text{Mg}^{2+}$ (0.01% each). The plates were then placed under a UVA light (Uvasun 3000) for a between 50 seconds and 20 minutes to receive a standard dose of 250 kJ/m^2 of energy at 25°C. The energy output of the UVA source was assessed on a daily basis and the duration of exposure calculated using a computer-generated algorithm. After this the plates were washed in PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$. The conditioned medium was then put back on the plates before incubation for a further 6 hours. The cells were washed twice in ice-cold PBS, and then 1.5 ml of PBS was placed on the cells before they were scraped and then transferred to a plastic specimen tube. This was then centrifuged 1 minute. The supernatant was discarded and the cell pellet flash-frozen in an ethanol/dry ice bath before storing at -80°C.

The pellet of FEK₄ cells was re-suspended in 200 µl of standard lysis buffer. The samples were placed on ice for 5 minutes and then rotated at 4°C for a further 15 minutes, and finally centrifuged for 15 minutes. The supernatant, containing the cytoplasmic extract, was then transferred to a new tube.

2.4 Consent for the use of historically stored slides etc

Previous work within the department had been covered by a local consent agreement (1990). This agreement was specifically extended for this study. A further agreement was sought to cover the molecular analysis of tissue samples in IBD, this protocol allowed the use of stored human tissue i.e. paraffin block samples. Where resection specimens were used full consent was gained from the patients prior to their operation. It was stressed to the patient that taking part in the study was entirely optional and if they took part in the study their operation and management would not be altered in any way (see appendix II for patient information sheets).

3 Results

3.1 Optimisation of immuno-staining conditions for HO-1 and HO-2

Standardisation of the optimal conditions for the HO antibodies was conducted on normal colon and terminal ileal resection specimens. These specimens had been harvested from the colonic end of a right hemi-colectomy specimen performed to resect a caecal carcinoma. The normal tissue margins were at least 7 cm distant from the cancer. The challenge when developing an immuno-staining technique is to maximize the specific staining of the protein of interest while minimizing the non-specific background staining. The optimum concentration of primary antibody is that which gives a clear colour change specifically identifying the target protein without background staining. There is an inverse relationship between the incubation time and antibody concentration required to achieve optimal conditions. The functional affinity of the antibody refers to the time taken for the antibody to reach equilibrium with the tissue antigen. Thus antibodies of high functional affinity need relatively little time to achieve a steady state with the tissue under analysis. The time to reach equilibrium is also temperature dependent. The conditions which were altered to find optimal processing included: (a) primary antibody concentration, (b) temperature of the primary antibody incubation, (c) duration of the primary antibody incubation, (d) the addition of a blocking stage to reduce non-specific background staining prior to the primary antibody and finally, (e) varying the concentration of the blocking solution.

The first set of conditions used was as per the ChemMate protocol. Primary antibody was applied for 30 minutes at room temperature. A range of HO-1 primary antibody concentrations were employed: 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1000, 1:1500. In Figure 12 two of these concentrations are shown. Both these slides show crystal artefacts from haematoxylin. Haematoxylin precipitation occurs at cold temperatures; subsequently the haematoxylin was always filtered before use. Figure 12 (a) shows heavy brown staining of almost all tissues, while Figure 12 (b) shows more selective uptake. The best signals were achieved at 1:1000 and 1:1500. At these concentrations there was granular staining in the cytoplasm of epithelial cells suggesting HO-1 presence in normal colonic epithelial cells.

The next variation in conditions to be explored was to extend the primary antibody incubation period to 16 hours (overnight) at 4°C. A range of primary antibody concentrations was used: 1:1000, 1:1500, 1:2000, and 1:3000. Figure 13 shows results at 1:1000 (A), 1:3000 (B) and a simple negative control (C, absence of primary antibody). Under these conditions 1:1000 appears optimal.

To reduce background staining in the negative control specimens a blocking stage was added. Before the primary antibody incubation the slides were bathed in 2 % BSA in TBS for 30 minutes. Figure 14 demonstrates the effect of the addition of the blocking solution 2% BSA in TBS for 30 minutes with a variety of primary antibody concentrations: 1:1000, 1:3000 and negative control (1:5000 performed but not shown). Under these conditions the 1:1000 primary antibody gave the clearest result and there was some improvement of the negative control.

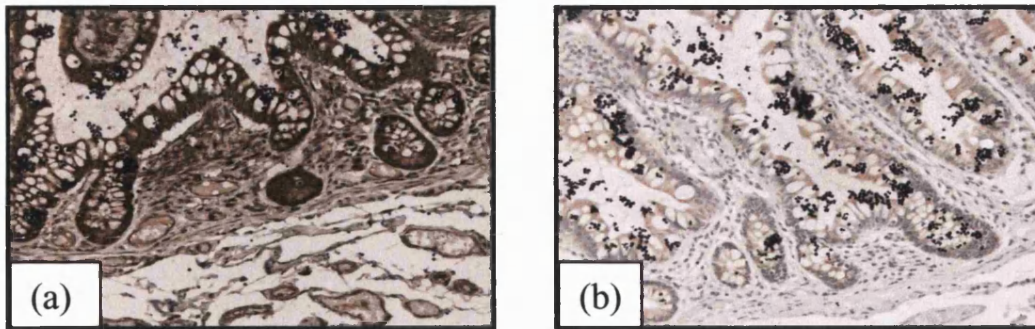


Figure 12 Optimizing HO immuno-staining I. First set of HO-1 immuno-staining conditions : primary antibody for 30 minutes at room temperature. Two concentrations of primary antibody are shown : (a) = 1 : 25, (b) = 1:1000 (NB full range of concentrations not shown). Original magnification 128x.

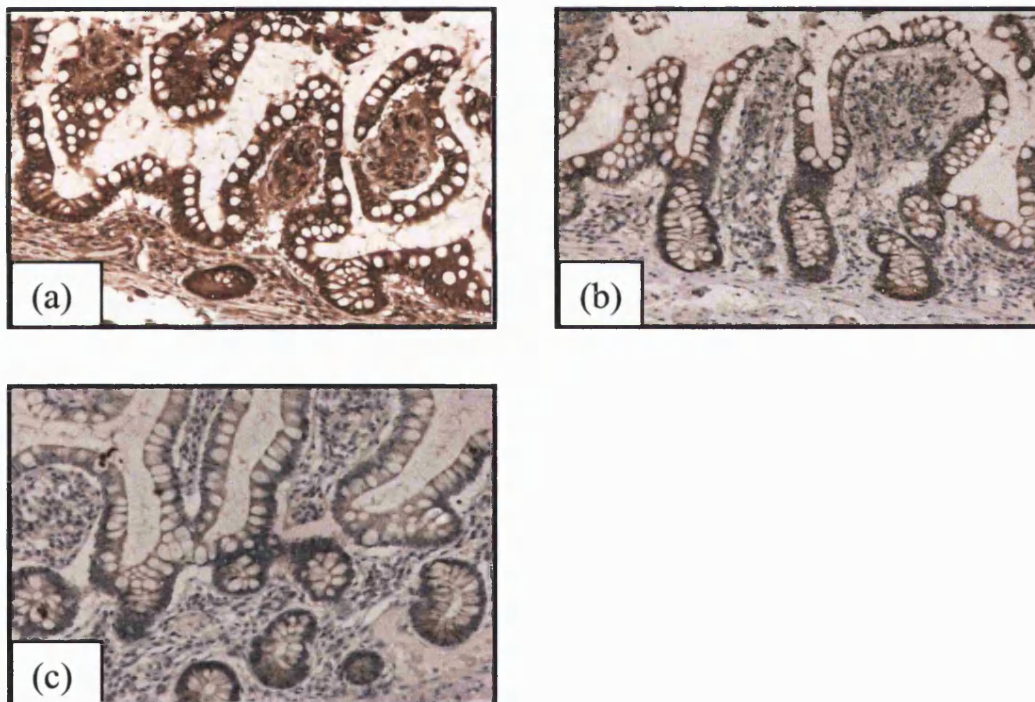


Figure 13 Optimizing HO immuno-staining II. Increased duration of primary antibody at 4°: HO-1 primary antibody overnight (16 hours) at 4°C. Concentration of primary antibody (a) = 1:1000, (b) = 1: 3000, (c) = control. Full range of concentrations not shown. Original magnification 128x.

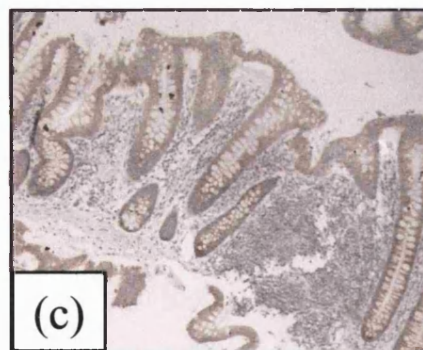
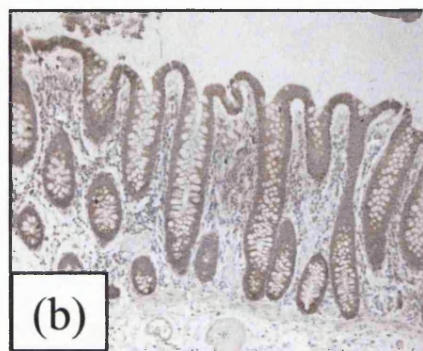
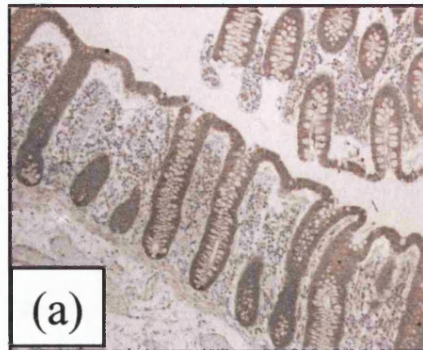


Figure 14 Optimizing HO immuno-staining III.

Additional blocking stage : an attempt to tone down control signal by adding a blocking stage with 2% BSA in TBS for 30 minutes prior to incubation with the primary antibody overnight at 4 ° C. (a) 1 in 1000, (b) 1 in 3000, (c) control. Some reduction in the background staining achieved by the blocking step, concentration of 1 in 1000 appeared to be optimal. Original magnification 128x.

In an attempt to further refine the negative control the period of blocking was extended to 1 hour with 5% BSA in TBS at pH 7.6. Figure 15 shows the results obtained using a primary antibody stored at 4°C overnight. Increasing the concentration of the BSA blocking solution from 5 to 10% (Figure 16), blocking for 1 hour and then applying primary antibody overnight at 4°C was then attempted as a final refinement. The higher concentration of blocking solution did not yield better results. The best conditions found used blocking with 5% BSA for 1 hour and TBS raised to pH 9.0 throughout, with a primary antibody concentration of either 1 in 500 or 1 in 1000 (Figure 17).

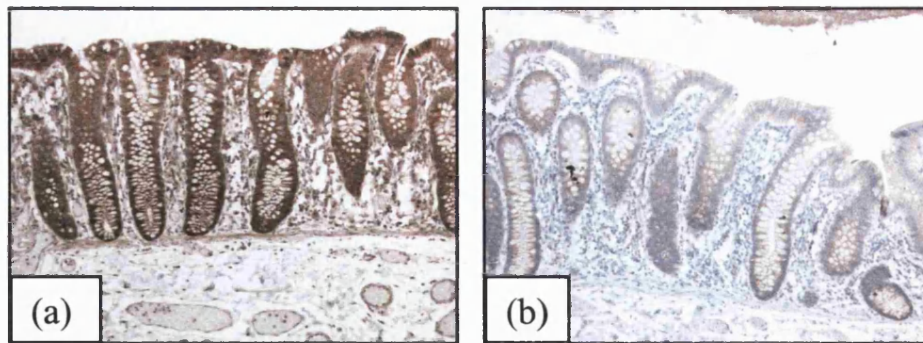


Figure 15 Optimizing HO immuno-staining IV.

Variation of the additional blocking stage to attempt to tone down control signal: 5% BSA in TBS (pH 7.6) for 1 hour then primary overnight at 4 °C. (a) 1 in 500, (b) control. Original magnification 128 x.

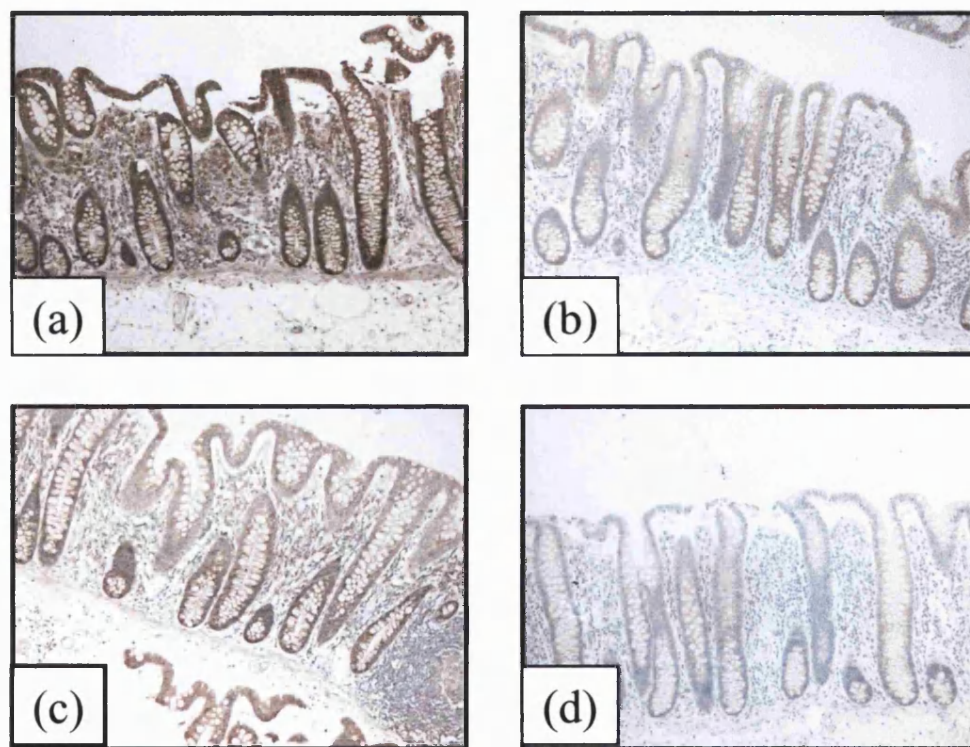


Figure 16 Optimizing HO immuno-staining V.

To investigate whether 10% BSA [(a) and (b)] better than 5% BSA [(c) and (d)]; (a) and (c) primary antibody concentration is 1 in 500 and (b) and (d) are simple negative controls. The better control with less background staining is (d) corresponding to the 5% BSA blocking solution. Original magnification 128 x.

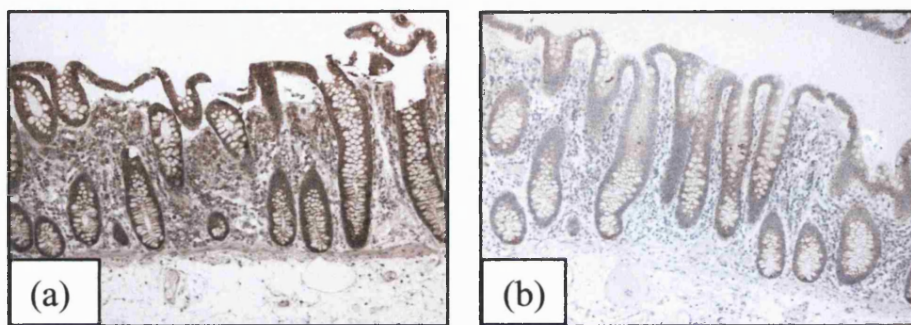


Figure 17 Optimizing HO immuno-staining VI.

Variation of the additional blocking stage to attempt to tone down control signal: 5% BSA in TBS (pH 9.0) for 1 hour then primary overnight at 4 ° C. (a) 1:500, (b) control.

Ultimately these were the best conditions.

Original magnification 128 x.

3.2 Normal colonic and terminal ileal expression of HO proteins

demonstrated by immuno-histochemistry

Having optimised the immuno-staining conditions for HO in human GI tissue a series of normal GI tissue sections were analysed. Immuno-staining was conducted on both full thickness resection specimens and also biopsy samples. Immunostaining was first undertaken in normal tissue to establish the distribution of HO-1 and HO-2. The remaining figures of this chapter (Figures 18-23) show the immuno-reactivity of HO-1 and HO-2 in normal small bowel and colonic specimens. In these figures positive immuno-staining is demonstrated by brown staining for either HO-1 or HO-2 depending on the primary antibody used. The principal observation was the expression of both HO-1 and HO-2 in the epithelial cells and in some of the cells of the lamina propria.

3.2.1 Expression of HO-1 and HO-2 in the healthy terminal ileum

Figure 18 shows immuno-staining of healthy terminal ileum. Figure 18 (a) shows HO-1 expression (brown stain is positive for HO-1). Figure 18 (b) shows the expression of HO-2 and Figure 18 (c) is a simple control with the omission of the primary antibody. HO-1 and HO-2 are expressed in normal terminal small bowel in the cytoplasm of the epithelial cells. The nuclei of the epithelial cells line the basal margin of the cells (away from the lumen), they stain blue and appear not to express either HO-1 or HO-2. Within the lamina propria only some of the inflammatory cells express the HO proteins.

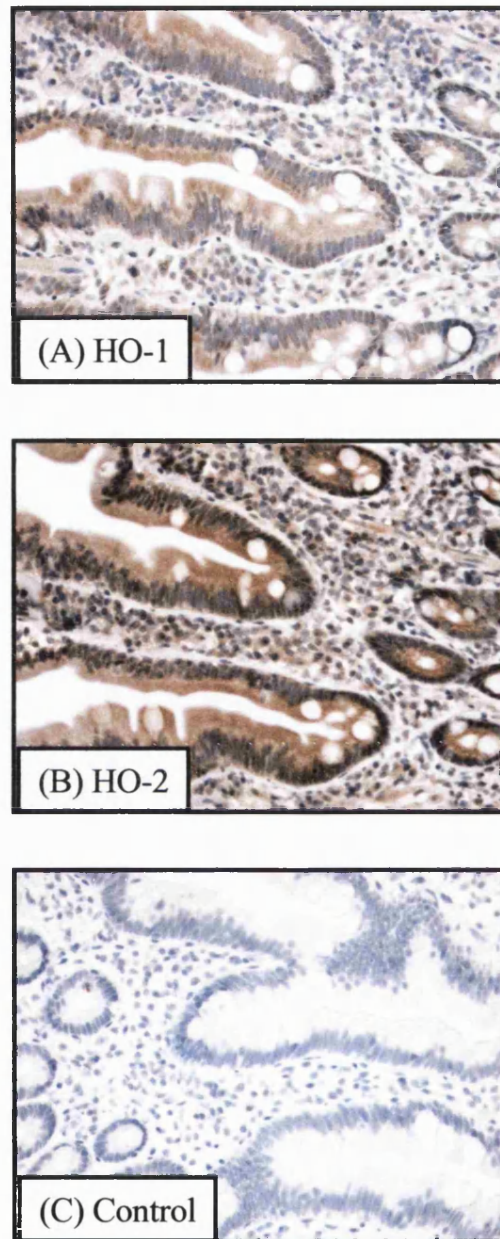


Figure 18 HO immuno-reactivity in the normal terminal ileum.

Immuno-staining for HO-1(A), HO-2(B) and control (C) (brown stain positive for protein expression). Demonstrating a similar distribution of HO-1 and HO-2 in the cytoplasm of epithelial cells and some inflammatory cells in the lamina propria. Slide (C) confirms no staining in the control conditions process without the primary antibody. Original magnification 128 x. Full conditions described in Materials and Methods and summarised in Figure 10.

3.2.2 Expression of HO-1 and HO-2 in the healthy colon.

Figure 19 demonstrates the expression of HO-1 (Figure 19 (a)) and HO-2 (Figure 19(b)) in the epithelium and lamina propria of normal colon. Immuno-staining demonstrated the expression of both HO-1 and HO-2 in the epithelial cells in a similar fashion to that seen in the terminal ileum with HO expression being in the cytoplasm but not in the nuclei. Inflammatory cells in the lamina propria also expressed both HO-1 and HO-2 in healthy colonic tissue.

3.2.3 Confirmation that macrophages express HO-1

The inflammatory cells expressing both HO-1 and HO-2 in the terminal ileum and the colon sections were identified as macrophages. The expression of HO in macrophages is demonstrated in slide Figure 20 (a) and (b). In Figure 20 (a) high power view shows strong HO-1 expression in the macrophages in normal lamina propria especially in the sub-epithelial region. Figure 20(b) shows a macrophage with strong HO-1 expression within a larger population of lymphocytes which do not express HO-1. The macrophage has a characteristic morphology easily recognised by an experienced histopathologist. Further confirmation that this cell population represented macrophages was gained by using a macrophage specific immuno-stain α -CD68. Figure 21 (a) shows immuno-staining for HO-1 in normal colon with expression in the epithelial cells and in inflammatory cells in the sub-epithelial cells of the lamina propria. Figure 21 (b) employs α -CD68 stain selective for macrophages and confirms that the sub-epithelial cells expressing HO-1 are macrophages, slide processed at University College London.

It would appear that the expression of HO-2 in the lamina propria occurs in a wider variety of cell types than that of HO-1.

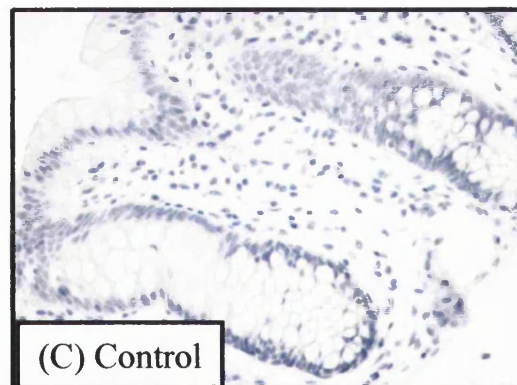
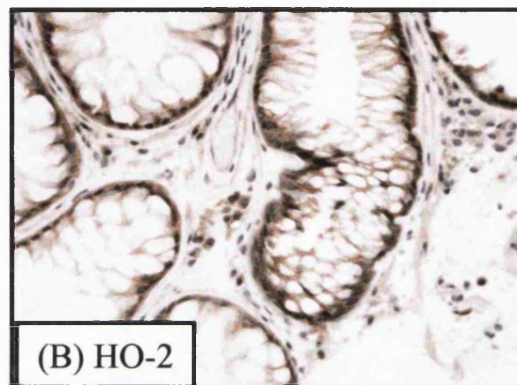
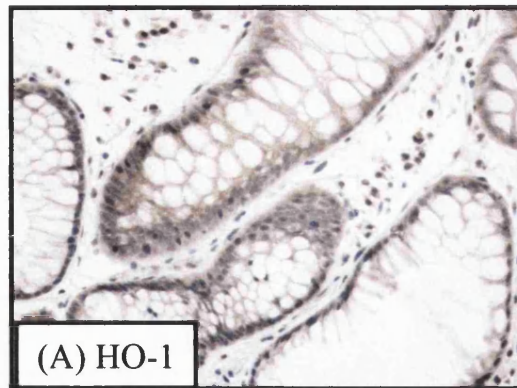


Figure 19 HO immuno-reactivity in the normal colon. Immuno-staining for HO-1(A), HO-2(B) and control (C) (brown stain positive for protein expression). Demonstrating a similar distribution of HO-1 and HO-2 in the cytoplasm of epithelial cells and some inflammatory cells in the lamina propria. Slide (C) confirms no staining under control conditions (processed without the primary antibody). Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

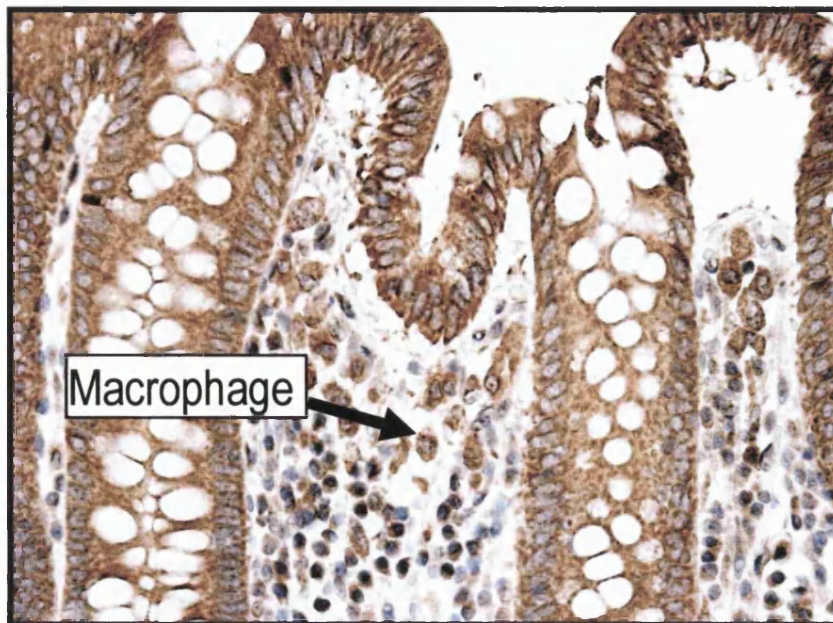


Figure 20a HO-1 expression by macrophages in the normal colon Brown stain positive for HO-1 in normal colon showing HO-1 expression in macrophages in the lamina propria. Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

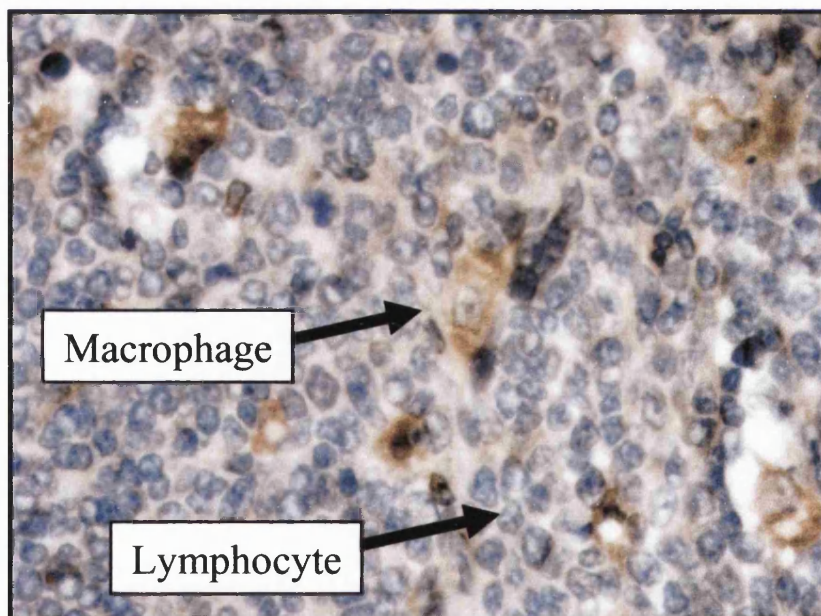


Figure 20b Small Bowel HO-1 immuno-reactivity (brown stain positive for HO-1) showing macrophages expressing HO-1, lymphocytes stain blue i.e. no HO-1 expression. Original magnification 320x. Full conditions described in Materials and Methods and summarised in Figure 10.

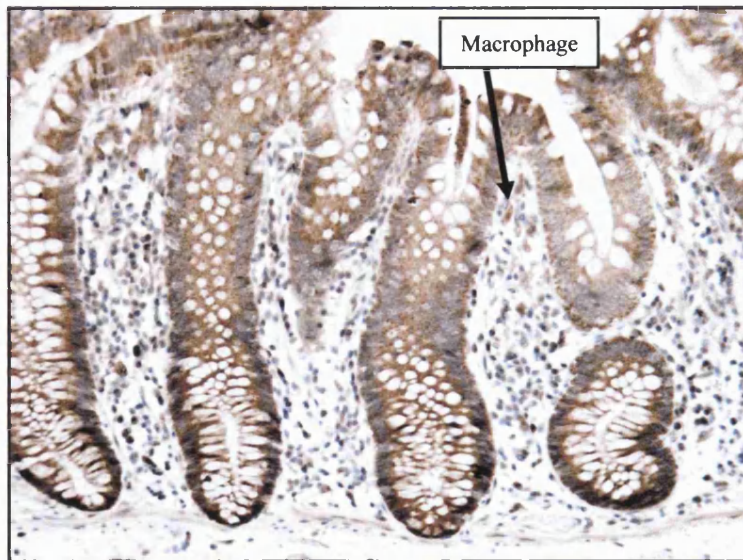


Figure 21a Normal colonic biopsy immuno-reactivity for HO-1 (brown stain positive for HO-1) showing HO-1 expression in epithelial cells cytoplasm and macrophages in the lamina propria. Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

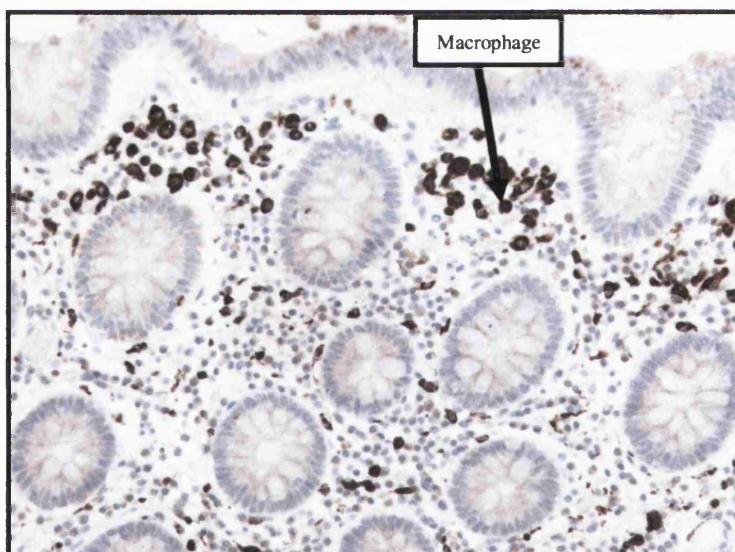


Figure 21b Normal colonic biopsy α -CD68 immuno-reactivity. This stain is selective for macrophages and demonstrates the sub-epithelial distribution of macrophages within the lamina propria, (the same distribution as the cells that express HO-1. Original magnification 128x.

3.2.4 HO-1 expression in deeper structures

Figures 22 (a) and 22 (b) are of neurological tissue within the submucosa of the colon. Figure 22 (a) shows an H+E stain of a submucosal plexus. Immunostaining confirmed the expression of HO-1 in the parasympathetic ganglion. The only previous study of HO expression in human GI tract included only superficial biopsy specimens, and therefore did not demonstrate expression of HO-1 in such deep structures (Barton 2003). The current work is therefore the first demonstration of HO-1 in human GI parasympathetic ganglia. Other deep sections also showed the expression of HO-1 in the endothelium of blood vessel walls (Figure 23 (a)). Figure 23 (b) provides a high power view of normal colonic epithelium showing the granular brown staining indicative of cytoplasmic expression of HO-1. The nuclei (stained blue) are seen at the basal aspect of each of the epithelial cells.

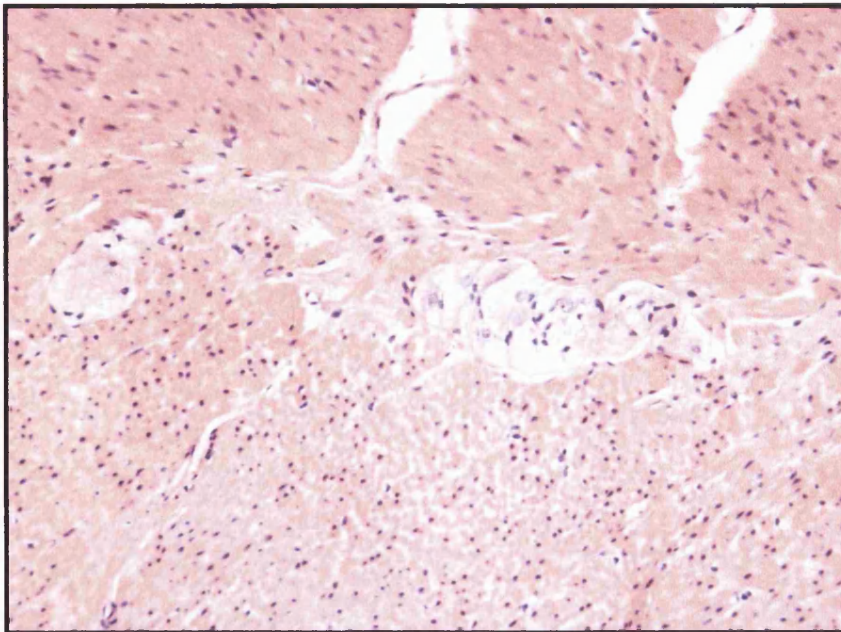


Figure 22a Haematoxylin and eosin stain of neuronal tissue within the submucosa of the colon. Original magnification 128x..

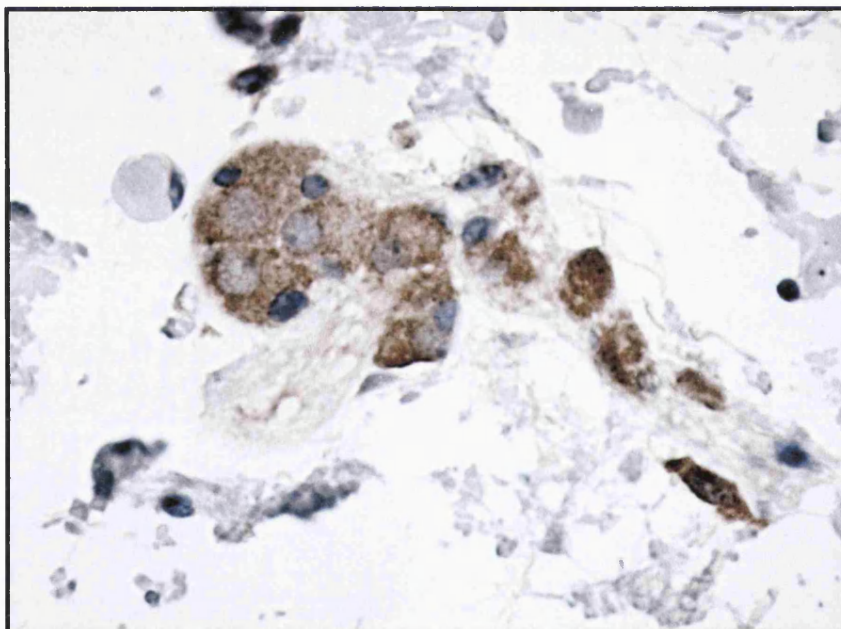


Figure 22b Parasympathetic ganglion shows HO-1 expression. HO-1 immuno-reactivity in normal colon (brown stain positive for HO-1) showing HO-1 expression in a parasympathetic ganglion. Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

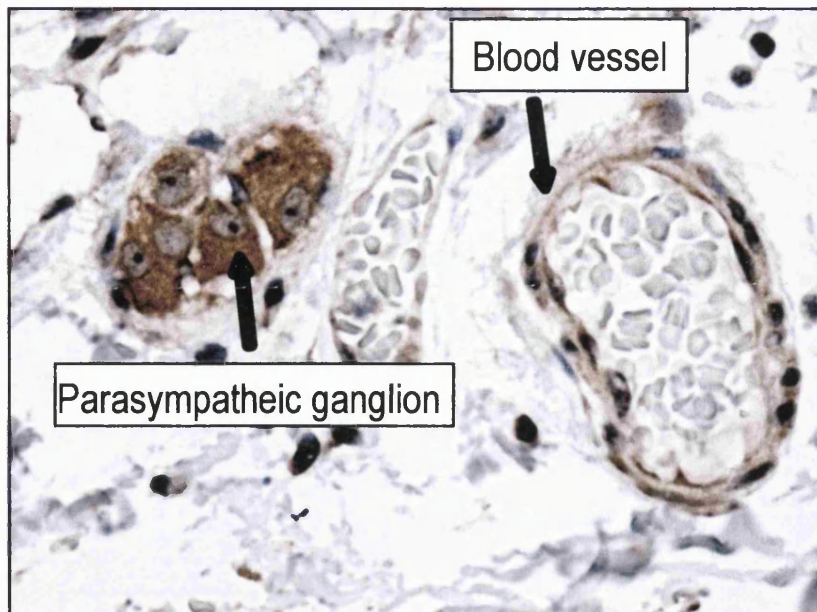


Figure 23a HO-1 immuno-reactivity in a colonic resection specimen showing submucosal structures : parasympathetic ganglion cells and endothelial cells lining the blood vessel stain positive for HO-1, showing expression of the protein Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

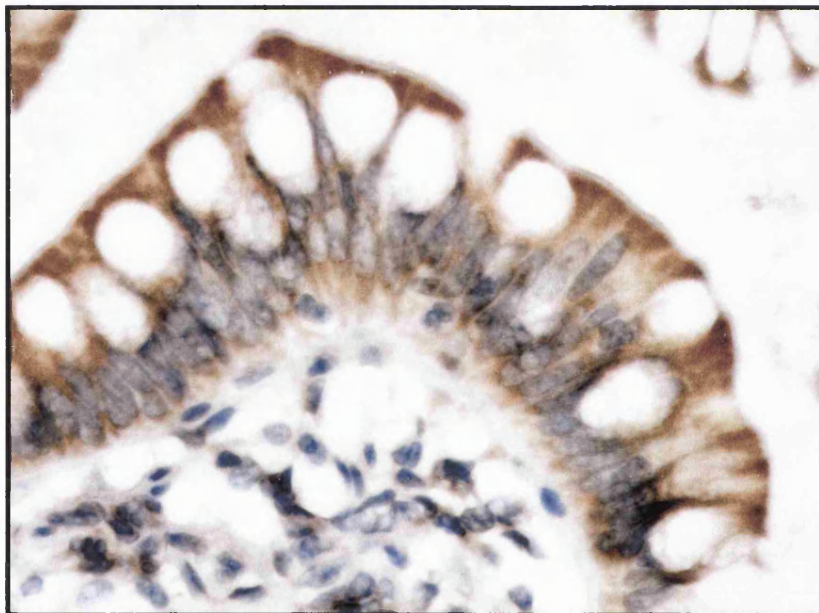


Figure 23b High power magnification showing HO-1 expression in the cytoplasm of colonic epithelial cells. Original magnification 320 x. Full conditions described in Materials and Methods and summarised in Figure 10.

3.3 IBD results

To test the hypothesis that differences in HO-1 could contribute to the development of IBD a comparison of the expression of HO in inflammatory bowel disease with that in normal tissue was undertaken. Twenty-eight sets of biopsies from normal colon were compared with 22 biopsies from cases with IBD. A summary of donor details is shown in Appendix III. The scoring system is described in detail in the methods section. The slides were processed in mixed batches from cases with IBD alongside those from healthy colon so that minor difference in slide preparation technique would not affect comparison between groups. The intensity of immuno-staining for HO-1 and HO-2 was scored for both the epithelium and the lamina propria. The complete data set for normal colon and IBD are shown in tables 6 (normal colon) and table 7 (IBD). The mean scores are shown in table 8. The principal investigator MF scored all slides. A subset of the slides was independently scored by NR to validate the inter observer variation; there was a 78% agreement between the two observers.

Mean intensity scores for immuno-staining for HO-1, normal compared to IBD

Intensity of staining scale: Table 8 . Also see appendix IV for median scores.

0 = no stain, 1 = medium intensity, 2 = strong intensity (shown in Figure 9)

	Normal n = 28	IBD n = 22	MWU
Control	0.25	0.13	
HO-1 epithelial	1.62	1.78	p = 0.143
HO-1 lamina propria	1.66	1.88	p = 0.03
HO-2 epithelial	1.83	1.86	
HO-2 lamina propria	1.83	1.95	

Table 6 Normal colon immunostaining intensity scores

		MF	MF	NR	NR	MF	MF
Sample	Control	HO-1 Epith	HO-1 LP	HO-1 Epith	HO-1 LP	HO-2 Epith	HO-2 LP
50	0	2	1			2	2
51	0	1	1	1	1	2	1
52	0	2	2	2	2	2	2
53	0	2	2	2	1	2	2
54	0	1	2	2	2	2	2
55	0	1	1	1	1	1	1
56	0	1	1	0	0	2	2
57	0	1	1	1	0	2	2
58	0	1	1	1	1	1	1
59	0	2	2	2	2	2	2
60	0	2	2	2	2	1	1
61	0	2	2			2	2
62	0	2	2			2	2
63	0	2	2			2	2
64	0	1	1			2	2
65	1	2	2			2	2
66	0	2	2			2	2
67	0	1	2			2	2
68	0	2	2			2	2
69	0	1	2			1	2
70	0	1	1			1	1
71	1	2	2			2	2
72	0	1	2			1	2
73	2	2	2			2	2
74	0	1	1			2	1
75	1	2	1			2	2
76	1	2	2			2	2
77	1	2	2			2	2

Epith = Epithelial cell score
LP= Lamina propria score

MF scored by Dr Feeney
NR scored by Dr Rooney

Table 7 IBD immunostaining intensity scores

		MF	MF	NR	NR	MF	MF
Sample	Control	HO-1 Epith	HO-1 LP	HO-1 Epith	HO-1 LP	HO-2 Epith	HO-2 LP
21	0	2	2	2	2	1	1
22	0	2	2	2	2	2	2
23	0	1	2	2	1	2	2
24	0	2	2	2	2	2	2
25	0	2	2	2	2	2	2
26	0	1	1	1	2	1	2
27	0	1	2	1	2	1	2
28	0	1	1	1	1	2	2
29	0	2	2	2	2	2	2
30	0	2	2	2	2	2	2
31	0	2	2			2	2
32	0	2	2			2	2
33	0	2	2			2	2
34	0	2	2			2	2
35	0	1	2			2	2
36	1	2	2			2	2
37	0	2	2			2	2
38	0	2	2			2	2
39	0	2	2			2	2
40	1	2	2			2	2
41	1	2	2			2	2
42	0	2	2			2	2

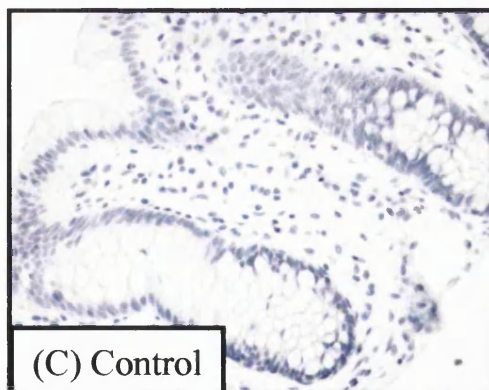
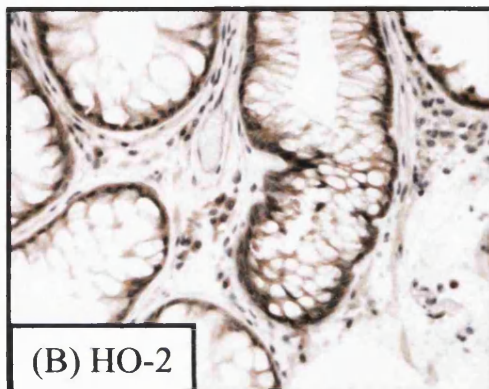
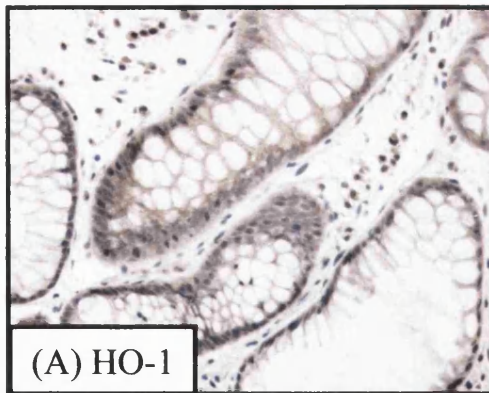
Epith = Epithelial cell score
LP= Lamina propria score

MF scored by Dr Feeney
NR scored by Dr Rooney

The intensity of immuno-staining for HO-1 was markedly increased in the lamina propria (LP) of the biopsies from cases of IBD. The difference in staining intensity between normal LP and the LP of IBD is significant with a p value < 0.05 (actual MWU test value $p=0.03$); the KW for LP scores was 0.048. This is illustrated by two representative cases shown in Figure 24 and 25; the biopsies from the cases of IBD are in the right hand vertical column. Immuno-staining for HO-1 shows that HO-1 expression occurs in macrophages. These cells were identified initially by their characteristic morphology. The distribution of the HO-1 to the macrophages was confirmed by the additional immuno-staining with α -CD68 specific for macrophages see Figure 26 (a) and 26 (b). This study did not measure the enzymic activity of the HO-1.

The HO-1 immuno-staining in the epithelium was also higher in IBD than in healthy colon biopsies. However this difference did not reach statistical significance: KW for epithelial scores 0.78 and MWU test p value was 0.143 therefore the null hypothesis that there is no difference was accepted. HO-2 (the constitutive form of the enzyme) was expressed in both normal tissue and that from IBD; however these data were not analysed for statistical significance. Figure 27 (a) and (b) show the expression of HO-1 in a case of Crohn's disease, a lymphoid aggregate being clearly seen at the base of the glands. Interestingly the expression of HO-1 seems to be particularly high in the cells forming the centre of the aggregate, possibly reflecting the degree of oxidant stress in this region. Figure 27 (c) shows a crypt abscess with the HO-1 expression being high both in the epithelial layer and in the lamina propria surrounding the crypt abscess.

Normal colon



Ulcerative Colitis

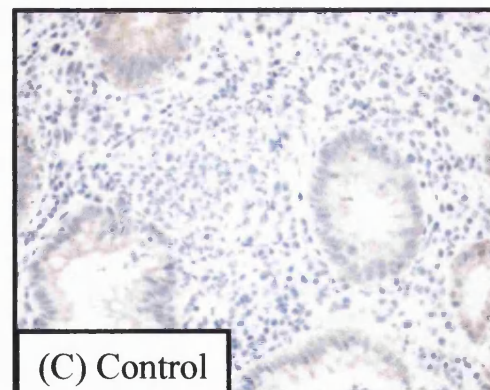
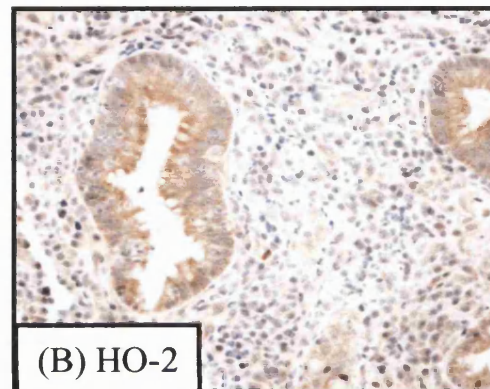
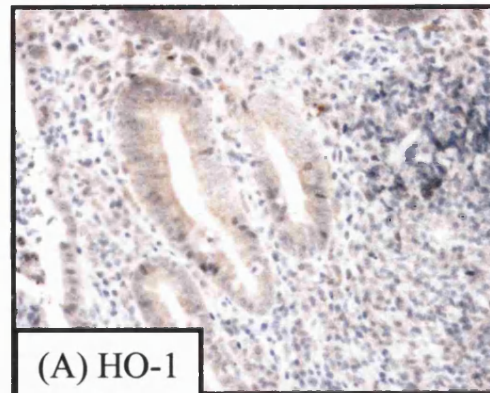


Figure 24 HO immuno-reactivity in normal colon (on the right) compared with UC (on the left). Brown stains positive for protein expression of (a) HO-1, (b) HO-2 and (C) control. These slides show that HO-1 and HO-2 are expressed in the epithelial cells and the inflammatory cells of the lamina propria of both normal colon and that of UC. Increased staining in the LP of IBD compared to normal colon reached statistical significance. Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

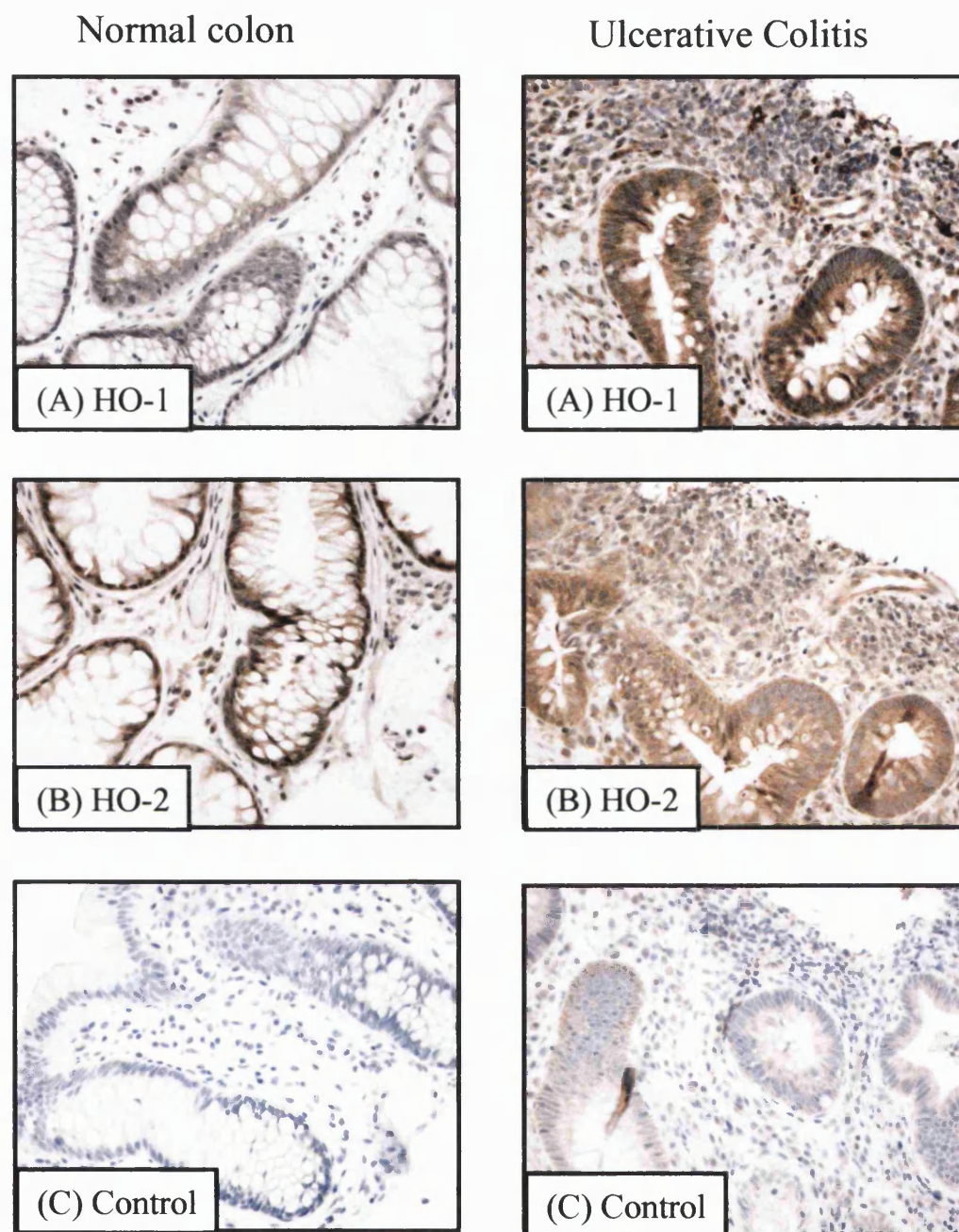


Figure 25 HO immuno-reactivity in normal colon (on the left) compared with UC (on the right) a second example. Brown stains positive for protein expression of (a) HO-1, (b) HO-2 and (C) control. These slides show that HO-1 and HO-2 are expressed in the epithelial cells and the inflammatory cells of the lamina propria of both normal colon and that of UC. Increased staining in the LP of IBD compared to normal colon reached statistical significance. Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

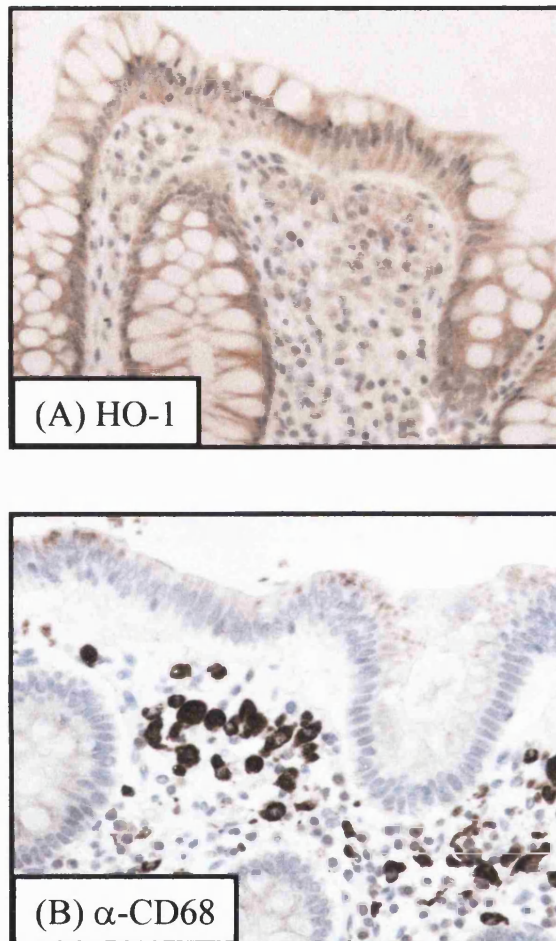


Figure 26 Macrophages express HO-1 in UC, (A) immuno-reactivity for HO-1 showing staining in the epithelial cell cytoplasm and macrophages in the sub-epithelial region. Full conditions described in Materials and Methods and summarised in Figure 10. (B) immuno-stain for α CD68 confirming that the cells in this region are macrophages. Original magnification 128 x.

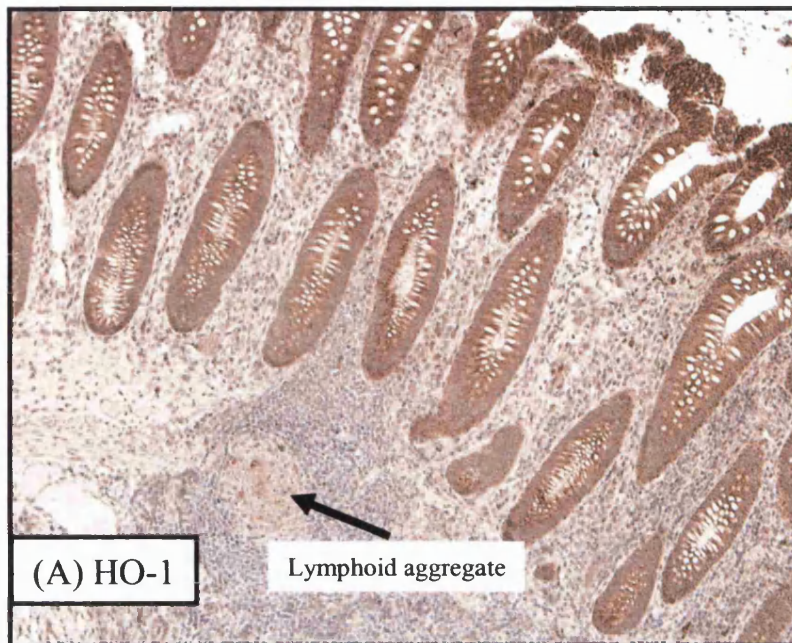


Figure 27a HO-1 expression in inflammatory cells within a lymphoid aggregate in a case of CD (the lymphoid aggregate is in the lamina propria just superficial to the muscularis mucosae). Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

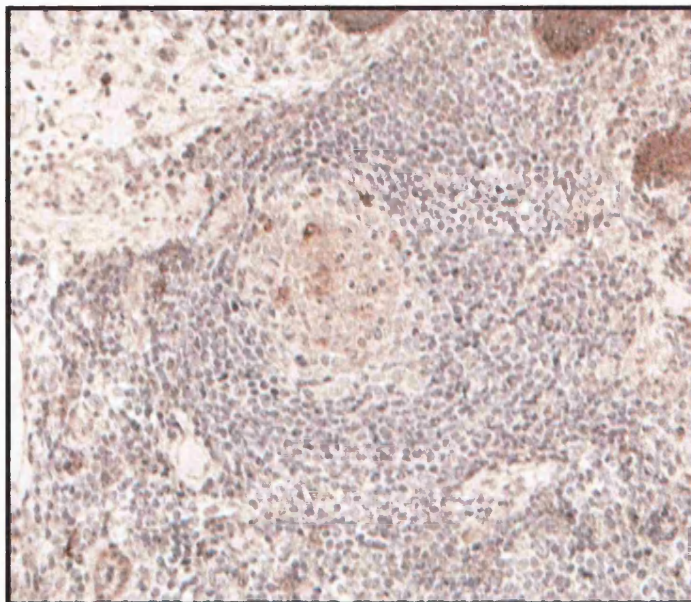


Figure 27b Lymphoid aggregate positive for HO-1 (enlarged from from slide 27a), showing HO-1 expression in the inflammatory cells. Original magnification 128x.

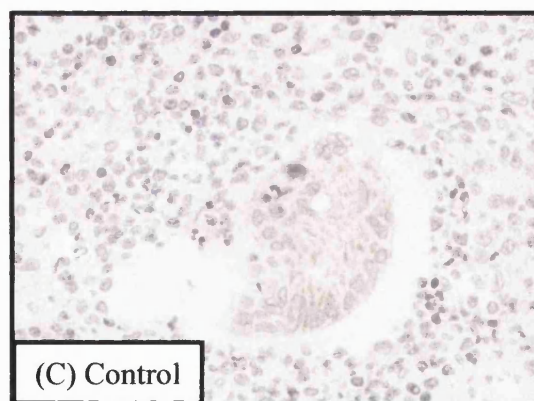
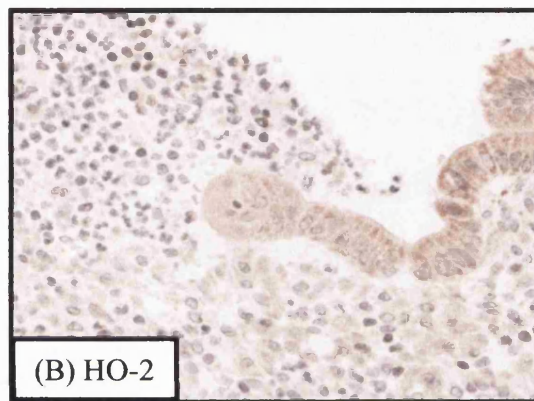
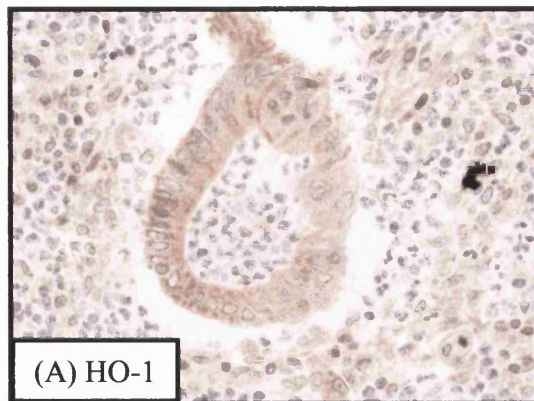


Figure 27c HO expression in the region of a crypt abscess. HO immuno-reactivity in a biopsy from a case of Crohn's disease. Immuno-staining for HO-1(A), HO-2(B) and control (C) (brown stain positive for protein expression). This slide demonstrates a crypt abscess. Original magnification 200x. Full conditions described in Materials and Methods and 111 summarised in Figure 10.

3.4 Colorectal carcinoma results

Twenty-eight sets of biopsies from normal colon were compared with 16 sets of biopsies from cases with colorectal carcinoma. The intensity of immuno-staining for HO-1 and HO-2 was scored for both the epithelium and the lamina propria. A summary of donor details is shown in Appendix III. The complete data set of all the intensity scores are shown in tables 6, normal colon and table 9, CRC. The mean scores are shown in table 10.

Table 10 The mean intensity for immuno-staining for HO-1 comparing normal colon with colorectal carcinoma. See Appendix IV for median values.

Scale 0= no stain, 1 = medium stain, 2 = strong stain (See Figure 9)

	Normal	Colorectal cancer	MWU
	n= 28	n= 16	
Control	0.25	0.56	
HO-1 epithelial	1.62	1.85	p = 0.04
HO-1 Lamina propria	1.66	1.88	p = 0.103
HO-2 epithelial	1.83	1.93	
HO-2 Lamina propria	1.83	1.93	

The intensity of HO-1 staining was greater in the biopsies of CRC (colorectal carcinoma) than in normal colonic tissue: this is illustrated in Figure 28 and 29.

This difference was demo

Table 9 Colorectal cancer immunostaining intensity scores

		MF	MF	NR	NR	MF	MF
Sample	Control	HO-1 Epith	HO-1 LP	HO-1 Epith	HO-1 LP	HO-2 Epith	HO-2 LP
1	1	2	2	2	1	2	2
2	0	1	1	1	1	2	2
3	0	2	2	2	1	2	2
4	0	2	2	1	1	2	2
5	0	2	2	2	2	2	2
6	1	2	2	2	0	2	2
7	1	2	2	1	2	1	1
8	0	2	2	2	1	2	2
9	0	2	2	2	2	2	2
10	1	2	2	2	2	2	2
11	0	1	1			2	2
12	2	2	2			2	2
13	1	2	2			2	2
14	1	2	2			2	2
15	1	2	2			2	2
16	0	2	2			2	2

Epith = Epithelial cell score
LP= Lamina propria score

MF scored by Dr Feeney
NR scored by Dr Rooney

nstrated in both the epithelium and in the lamina propria. The difference in epithelial staining reached statistical significance as the MWU value was < 0.05 (MWU = 0.04). The difference in staining of the lamina propria did not reach significance MWU $p= 0.103$. The control score for the colorectal carcinoma is higher than that for the normal tissue and it is possible that the higher background staining in this group could account in part for the observed differences in the HO-1 stained specimens. The KW value for all colonic epithelial scores was greater than 0.05 (KW= 0.078).

Figure 28 demonstrates the differences seen. The biopsies of normal tissue in the left hand column are from normal colon and those on the right are from a colorectal carcinoma. In the case illustrated both sets of biopsies were taken from the same patient during a single colonoscopic examination. The biopsies were processed on the same slide. They have therefore been subjected to exactly the same immuno-staining conditions, so the differences seen are likely to represent real effects. This clearly shows that the intensity of immuno-staining for HO-1 is markedly increased in the epithelial cells in colorectal cancer when compared to those in normal colonic tissue. Figure 29 illustrates another case of colorectal cancer. Again the epithelial cells of the colorectal cancer show high intensity of HO-1 immuno-staining suggesting high levels of HO-1 expression.

Normal tissue

Colorectal Carcinoma

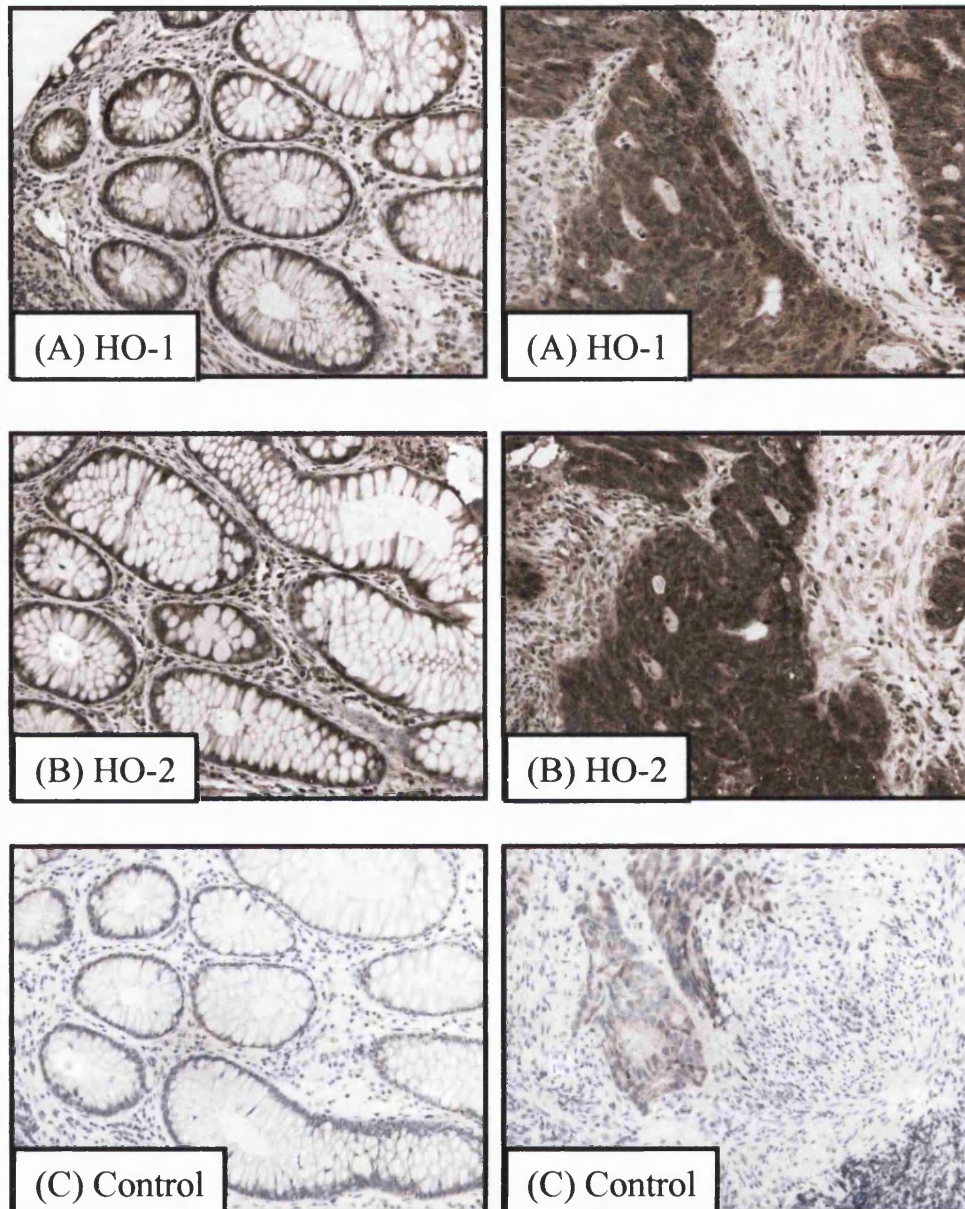


Figure 28 HO immuno-reactivity: On left normal tissue, on the right a colonic carcinoma (both sets of biopsies came from the same patient and were processed on the same slide). Demonstrating higher expression of HO-1 and HO-2 in tissue of the carcinoma as seen by the more intense brown stain. Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

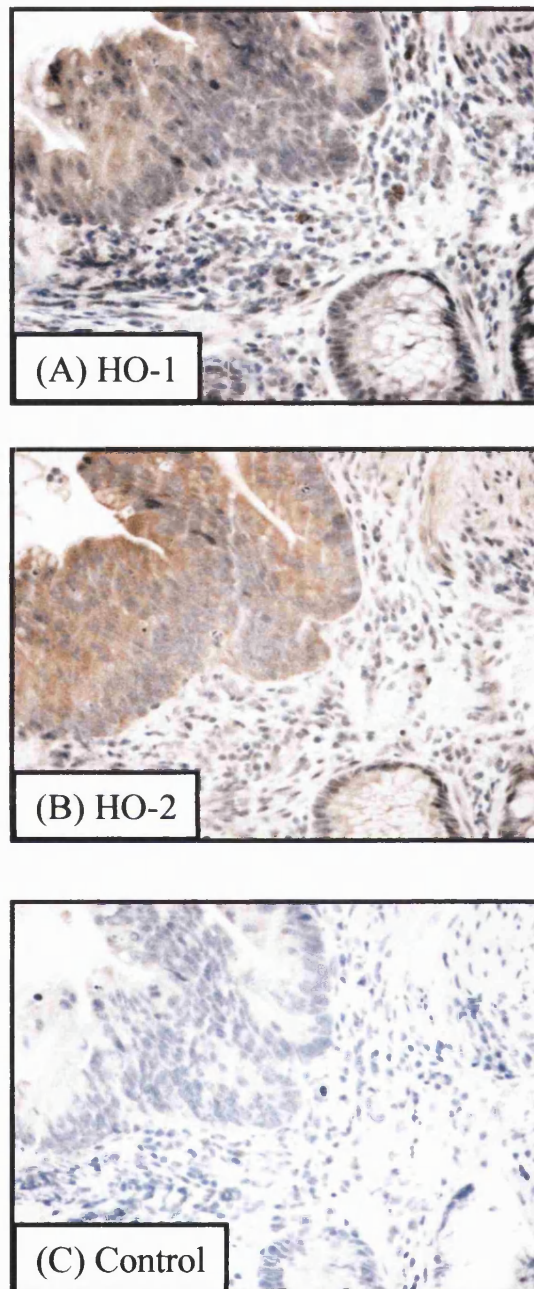


Figure 29 HO immuno-reactivity a colonic carcinoma, this set of biopsies show increased HO-1 and HO-2 in the epithelial cells (brown stain). Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

3.5 Protein production by the HT-29 colonic epithelial cell line

To investigate the expression of HO-1 in epithelial cells in another way, the cancer cell line HT-29 was used. HT-29 cells originate from a single colonic adenocarcinoma. This cell line is widely used to study the behaviour of colonic epithelial cells in vitro. The Western blotting technique was used to probe for the presence of HO-1 protein, to validate the observations of protein expression that had been demonstrated by the immuno-staining experiments. This cell line has been extensively used to model the action of inflammatory mediators on colonic epithelial cells. Pro-inflammatory cytokines such as $\text{IFN}\alpha$ have been shown to induce the expression of COX-2 while the anti-inflammatory cytokine IL-13 abrogates this effect (Weaver 2001). In the present study the HT29 system was first investigated by a series of experiments to confirm COX-2 production prior to exploring the effect of $\text{TNF}\alpha$ and IL-13 on the production of HO-1.

3.5.1 COX-2 protein production in the HT-29 cell line

The action of $\text{TNF}\alpha$ was first studied on HT29 cells. Figure 30 (a) shows a COX-2 Western blot of a an experiment stimulating HT29 cells with increasing concentrations of $\text{TNF}\alpha$. The cells were harvested 6 hours after stimulation with $\text{TNF}\alpha$. Figure 30 (a) demonstrates that at 6 hours a COX-2 protein signal is induced by $\text{TNF}\alpha$, and that this signal becomes progressively stronger with increasing concentrations of $\text{TNF}\alpha$. A strong signal was achieved with 100 ng/ml $\text{TNF}\alpha$ and in subsequent work in HT29 cells this was the standard concentration of $\text{TNF}\alpha$ used. The COX-2 signal corresponds to 74 kDa, and this experiment was run on a 7.5% SDS-PAGE gel. The time course of the action of $\text{TNF}\alpha$ on HT29

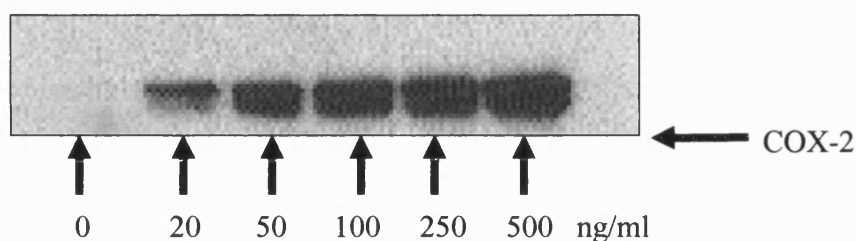


Figure 30a COX-2 protein expression by HT29 cells.

This Western blot demonstrates the COX-2 protein expression by HT29 colonic epithelial cells when stimulated by increasing concentrations of TNF α . This experiment shows a concentration gradient of TNF α at 0, 20, 50, 100, 250, 500 ng/ml. Six-well plates were used to culture the HT29 cells to 90% confluency, each well contained 3.5×10^6 cells with a protein yield of 1.5mg. Following stimulation the cells were incubated at 37 $^{\circ}$ C for 6 hours before harvest. This blot shows that in this cell line a good protein yield of COX-2 is achieved after stimulation with TNF α at a concentration of 100ng/ml. Representative of three experiments.

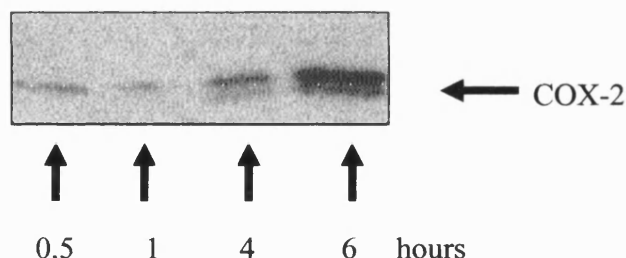


Figure 30b Increasing expression of COX-2 by HT-29 cells over time after stimulation with TNF α . This Western blot demonstrates the time course of COX-2 protein expression in HT29 cells when stimulated by TNF α at a concentration of 100 ng/ml. The cells were incubated at 37 $^{\circ}$ C and harvested at a series of time intervals: 0.5, 1, 4, and 6 hours after stimulation with TNF α . The strongest protein expression of COX-2 was achieved at an interval of 6 hours after stimulation with TNF α . Representative of two experiments.

cells is shown in Figure 30 (b). A weak signal is seen after 4 hours and by 6 hours the signal is easily seen. In subsequent work COX-2 protein signals were observed after harvesting at 6 hours or later. The COX-2 signal is seen as a doublet on Western blotting.

3.5.2 HO-1 protein production by the HT-29 cell line

Initially an in house HO-1 antibody was used to probe for the HO-1 protein. Even at high concentrations (1:50) this primary antibody did not give reliable results. A commercial antibody from Santa Cruz was bought (H-105, sc-10789) and this gave much better results. The same antibody had been used for the immunostaining work.

Positive controls for HO-1 protein were created in two ways:

- (a) Jurkat cells were stimulated with heme.
- (b) FEK cells were treated with UVA light to induce the production of HO-1 in this skin fibroblast cell line.

Figure 31 (upper blot) shows a Western blot for HO-1 protein in five different samples used as positive controls for HO-1. A lysate from Jurkat cells, which have been stimulated with heme occupies the first lane of the blot. The next four lanes have lysates from FEK cells treated with UVA. All of these samples demonstrate a protein band corresponding to a protein of molecular weight 32 kDa (HO-1). The protein yield from the FEK cells was lower than that from the Jurkat cells as indicated by the weaker signal for FEK cells on the Western blot. To produce enough protein to give reliable signal 8 dishes of FEK cells had to be

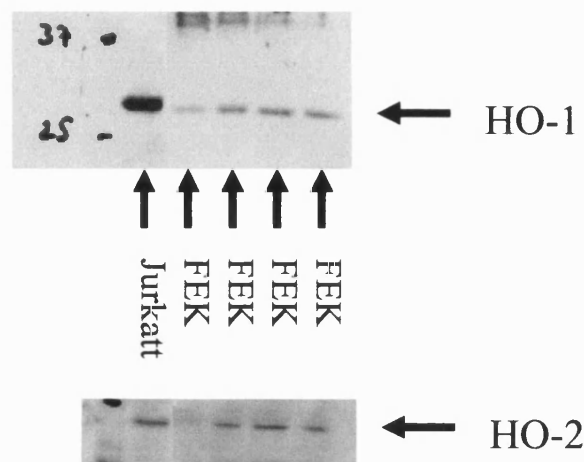


Figure 31 Western blot for HO-1 protein expression in positive control samples (upper blot). First lane derived by stimulating Jurkatt cells with heme. The other four lanes show HO-1 expression from the skin fibroblast cell line FEK stimulated by UVA(250 KJ/m²), then incubated at 37 °C for 6 hours before lysis. The lower blot demonstrates equal loading by probing for HO-2 the constitutive form of the enzyme. The sample in lane 2 shows a low protein yield for HO-2. Representative of 3 experiments.

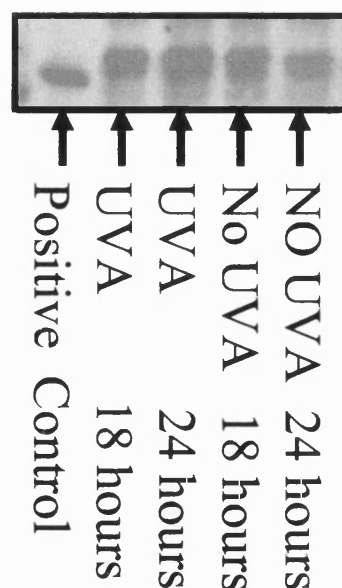


Figure 32 Western blot of HO-1 expression in HT29 cells, investigating the effect of UVA treatment on HO-1 expression. The HO-1 signal is slightly stronger in the cells treated with UVA than in those not treated with UVA. The HO-1 signal seen in the HT29s is at a marginally heavier weight than that of the controls (FEK cells) This may be related to a modification of the protein such as glycosylation. Representative of 3 experiments.

pooled. In the subsequent work the positive controls for HO-1 were either the Jurkat cell line stimulated with heme or the skin fibroblast cells exposed to UVA. Figure 31 (lower blot) shows equal loading of the samples in Figure 31 by re-probing with HO-2 as the primary antibody (sc-11361). NB HO-2 has a slightly larger molecular weight (36 kDa). HO-2 is a constitutive form of the enzyme and is also present in the FEK cells that have been exposed to UVA.

3.5.3 HO-1 expression is increased by UVA exposure

Figure 32 illustrates the effect of UVA stimulation on HT29 cells harvested at 18 and 24 hours after the exposure to UVA. The first two lanes are those from the light stimulated (250 kJ/mm^2) cells and the next two lanes are from those without UVA treatment. All the samples have produced HO-1. HO-1 production in those cells that have been exposed to UVA is marginally greater than those not exposed i.e. the resting level of HO-1 expression is increased by exposure to UVA.

3.5.4 HO-1 protein present in cells 1 hour post stimulation (Figure 33)

Figure 33 is an HO-1 Western blot showing the presence of HO-1 protein in HT29 cells less than six hours after treatment with $\text{TNF}\alpha$, there is a clear HO-1 signal at 1 hour post stimulation. The HT29 cells were stimulated with $\text{TNF}\alpha$ at a concentration of 100 ng/ml. HO-1 protein expression is thought to occur after 6-8 hours. This blot suggests that HO-1 is present in the cells prior to the influence of $\text{TNF}\alpha$. This result indicating that the colonic cell line HT29 is likely to express HO-1 under resting conditions supports the observations made by immunohistochemistry of colon biopsies. Immuno-staining showed expression of HO-1 in normal colonic epithelial cells.

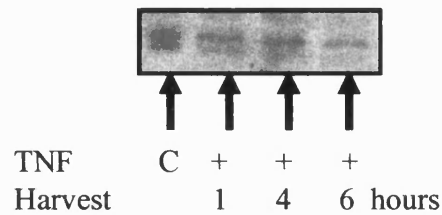


Figure 33 HO-1 expression in HT-29 cells stimulated by TNF α . This Western blot for HO-1 protein demonstrates the effect of TNF α (100 ng/ml) on HT29 cells. The positive control for HO-1 is marked as C(heme stimulated Jurkatt cells). HO-1 expression is present at 1, 4 and 6 hours post stimulation. This suggests that HO-1 is present prior to treatment with TNF α (in the resting state). Single experiment only.

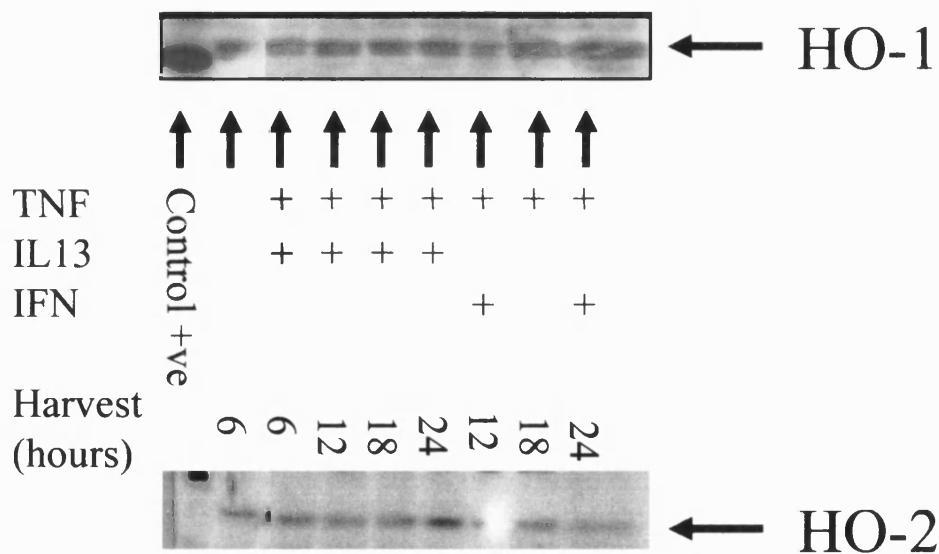


Figure 34 Western blot of HO-1 protein expression by HT29 cells stimulated by a combination of TNF α (100ng/ml), IFN γ (300U/ml) and IL-13 (50ng/ml). Cytokines were added at time 0 and the cells harvested at 6, 12, 18 and 24 hours. Presence of cytokine indicated by +. HO-1 expression is seen across all samples, independent of these cytokines. The equal loading blot probed for HO-2 is shown below. Representative of 3 experiments.

3.5.5 HO-1 expression by HT29 cells after cytokine stimulation

Figure 34 shows the result of mixed cytokine stimulation of HT29 cells (TNF α and IL-13). There are two notable features of this blot. Firstly the HO-1 signal in the HT29s is slightly higher than that in the controls, corresponding to a slightly larger molecular weight. This may be because the HO-1 protein is in someway slightly modified, for example, glycosylated or phosphorylated. Secondly, the HO-1 signal is strongly present across all lanes in the blot suggesting that HO-1 production is independent of cytokines to which the HT29s have been exposed. Cytokine exposure in this experiment did not increase HO-1 protein above basal levels. The HO-1 signal is present even in the un-stimulated sample harvested at 6 or 12 hours. All these samples are harvested after 6 hours. The HO-1 protein signal persists in cells to at least 24 hours. The presence of HO-1 in the un-stimulated cells is consistent with the observed expression of HO-1 in the resting colonic epithelium analysed by immuno-staining. Also in Figure 34 is a Western blot for HO-2 that demonstrates equal loading of the previous experiment: a faint signal of HO-2 can be seen across all lanes.

3.6 Results for oesophageal HO-1 and HO-2 immuno-staining

Twenty normal oesophageal sets of biopsies were compared with biopsies from 18 cases with Barrett's oesophagus cases. The intensity of staining for HO-1 and HO-2 was scored for both the epithelial region and the lamina propria. The complete data set of all immuno-staining intensity scores are shown in table 11 (normal oesophagus) and table 12 (Barrett's oesophagus). The mean scores are shown in table 13. In some oesophageal biopsies the sample disintegrated and the LP region was lost, in these cases no score was assigned. A summary of donor details is shown in Appendix III.

Table 13 Mean intensity scores for immuno-staining for HO-1

Intensity of staining scale: See Appendix IV for median values.

0 = no stain, 1 = medium intensity, 2 = strong intensity (shown in Figure 8)

	Normal oesophagus n = 20	Barrett's oesophagus n = 18	MWU test
Control	0	0.17	
Epithelial HO-1	1.45	1.82	p = 0.044
LP HO-1	1.4	1.88	p = 0.026
Epithelial HO-2	1.85	1.88	
LP HO-2	1.55	2.0	

The difference in epithelial staining in the two groups of oesophageal biopsies was striking. The expression of both HO-1 was much greater in the Barrett's oesophagus than in the normal oesophageal tissue (see Figure 35). A second case of Barrett's oesophagus is illustrated in figure 36, and shown alongside a normal

Table 11 Normal oesophageal immunostaining intensity scores

*Some biopsy samples disintegrated and lost the LP region, therefore no score could be assigned

		MF	MF	NR	NR	MF	MF
Sample	Control	HO-1 Epith	HO-1 LP	HO-1 Epith	HO-1 LP	HO-2 Epith	HO-2 LP
101	0	2	2	2	2	2	2
102	0	1	1	2	1	2	2
103	0	1	1	1	1	2	1
104	0	1	*	1	*	2	
105	0	1	1	2	1	2	1
106	0	1	*	1	*	2	
107	0	1	*	1	*	2	1
108	0	1	*	2	1	2	
109	0	2	2	2	*	2	2
110	0	2	*			2	
111	0	2	*			2	
112	0	2	2			2	2
113	0	1	*			2	
114	0	2	1			2	1
115	0	1	*			1	
116		1	*			1	
117	0	1	*			1	
118	0	2	*			2	
119	0	2	*			2	
120	0	2	2			2	2

Epith = Epithelial cell score
LP= Lamina propria score

MF scored by Dr Feeney
NR scored by Dr Rooney

Table 12 Barrett's oesophagus immunostaining intensity scores

		MF	MF	NR	NR	MF	MF
Sample	Control	HO-1 Epith	HO-1 LP	HO-1 Epith	HO-1 LP	HO-2 Epith	HO-2 LP
130	0	2	2	2	2	2	2
131	0	2	2	1	1	2	2
132	0	2	2	2	2	2	2
133	0	2	2	2	2	2	2
134	0	2	2	2	1	2	2
135	1	2	2	2	2	2	2
136	1	2	2	2	2	2	2
137	0	2	2	2	2	2	2
138	0	2	2	2	2	2	2
139	0	2	2	2	2	2	2
140	0	2	2	2	2	2	2
141	0	1				1	
143	0	1	1			2	2
144	0	2	2			2	2
145	0	1	1			2	2
146	0	2	2			1	2
147	0	2	2			2	2
149	0	2	2			2	2

Epith = Epithelial cell score
LP= Lamina propria score

MR scored by Dr Feeney
NR scored by Dr Rooney

HO-1

Control

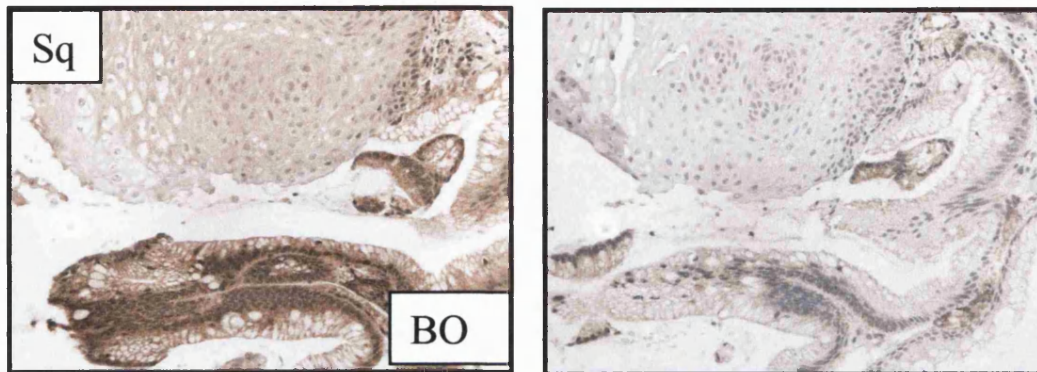


Figure 35 HO-1 immuno-reactivity of an oesophageal biopsy, HO-1 and simple control (absence of HO-1 primary antibody). Normal squamous epithelium (Sq) and neighboring Barrett's Oesophagus (BO) on the same slide. This slide demonstrates increased HO-1 expression in the BO tissue compared to normal squamous epithelium. Full conditions described in Materials and Methods and summarised in Figure 10.

Normal squamous oesophagus

Barrett's oesophagus

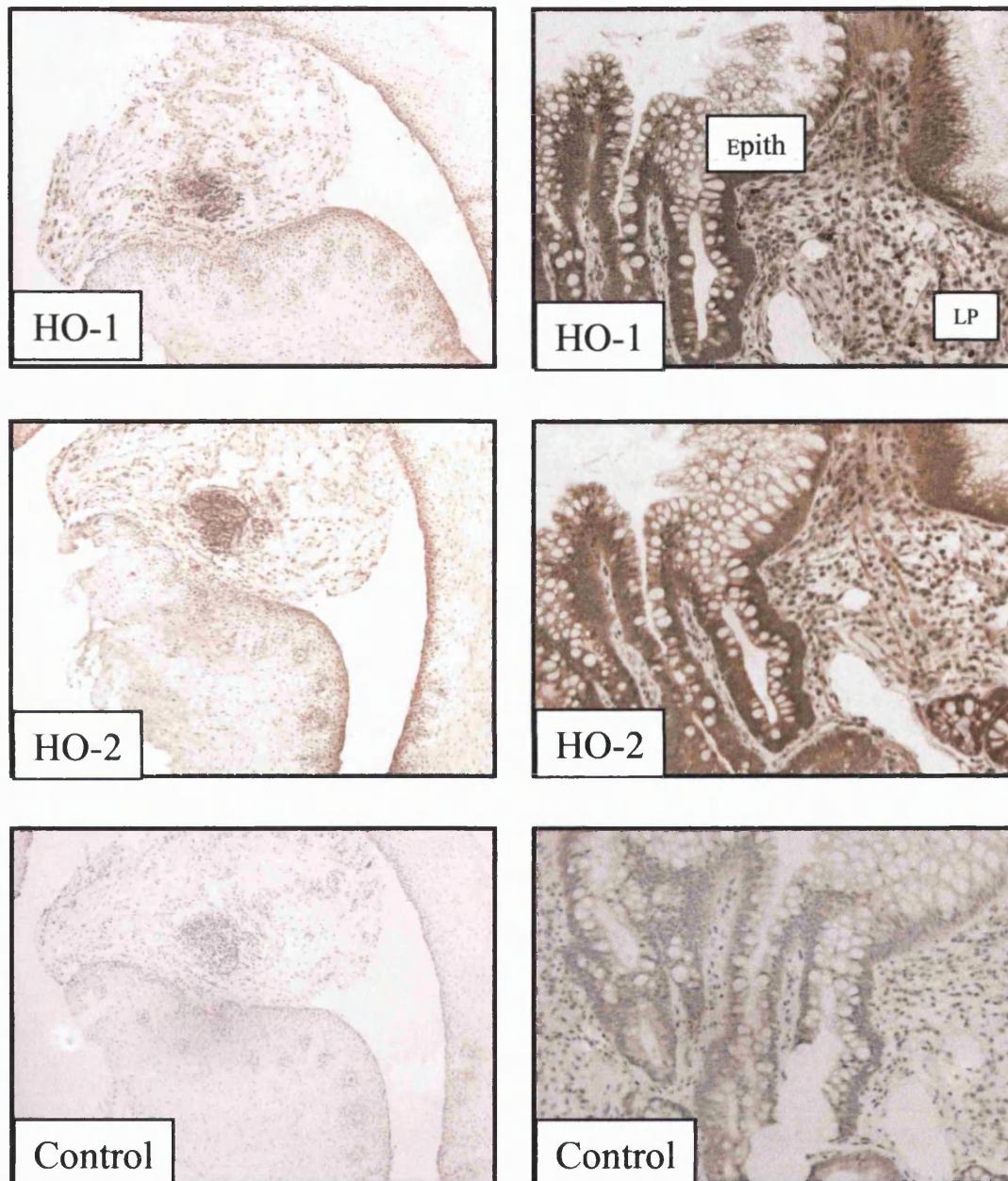


Figure 36 HO immuno-reactivity comparing normal oesophageal tissue with Barrett's oesophagus: on the left normal squamous epithelium, showing very little expression of HO-1 or HO-2, on the right a case of Barrett's oesophagus showing strong expression of both HO-1 and HO-2 in the columnar epithelial cells (Epith) and lamina propria (LP). Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

squamous biopsy. The average immuno-staining score are shown in table 13 (3 point scale). The greater expression of HO-1 in Barrett's oesophagus compared to normal squamous tissue occurred in both the epithelial cells and the cells of the lamina propria.

Comparisons between the two groups were made using the Mann-Whitney U test for unpaired data. Significance levels of $p < 0.05$ (two-tailed) were taken to be statistically significant. The differences found for HO-1 staining between normal and Barrett's oesophagus were significant by these criteria for both the lamina propria (MWU $p=0.026$) and the epithelium (MWU $p=0.044$) in the oesophagus. HO-2 expression was very similar in the epithelial cells of normal and Barrett's oesophagus. The expression of HO-2 was greater in the lamina propria of Barrett's oesophagus. These data were not analysed for statistical significance.

Figure 35 shows a transition point with normal squamous epithelium and Barrett's oesophagus on the same biopsy. This biopsy elegantly illustrates the differences of HO-1 expression seen in the two types of oesophageal tissue. The expression of HO-1 in the Barrett's oesophagus is very strong (score 2) whereas the expression in the normal oesophagus is much weaker (score 1). The simple negative controls (absence of primary antibody) are shown with score of 0. This biopsy illustrates the differences very clearly. Self evidently the preparation conditions within this biopsy were identical so differences in staining between the two types of oesophageal tissue are very likely to be real.

3.7 HO-1 expression by intestinal spirochaetes, a novel observation:

In one colonic biopsy specimen spirochaetes were identified by light microscopy of the H+E slide. An image of spirochaetes is illustrated in Figure 37 (Shah 2001). Immuno-staining using the rabbit polyclonal antibodies showed increased expression of HO-1 and HO-2. This has not previously been described. This observation was confirmed in one normal colonic biopsy and in one with mild IBD. The case of IBD is illustrated in Figure 38. The layer of spirochaete organisms clinging to the epithelial surface is easily seen in Figure 38, there is strong immuno-staining for both HO enzymes within the spirochaetes.

3.8 Expression of HO-1 in other gastro-intestinal malignancies

A small number of slide samples from other GI malignancies were also immuno-stained for HO. Figure 39 shows the results from a gastric adenocarcinoma and Figure 40 the results from a gastric carcinoma that had metastasised to the colon. In both cases there is strong expression of HO-1 and HO-2 in the cancerous epithelium.

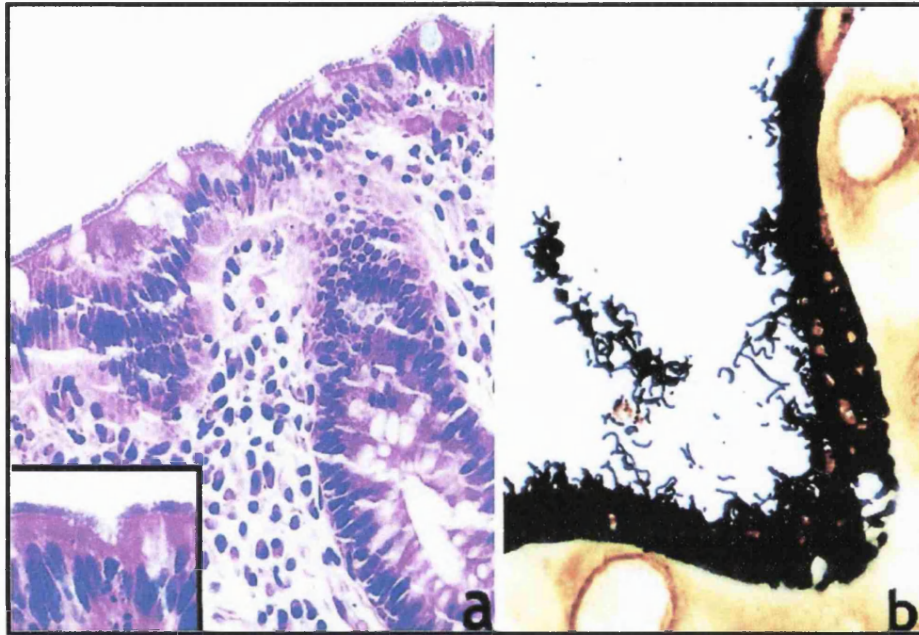


Figure 37 Intestinal spirochaetosis, image (a) is a standard H+E stain demonstrating the a false brush boarder, formed by the spirochaetes attached longitudinally to the surface of the epithelial cells. Inset bottom left is a high power magnification. (b) A high power magnification of the epithelial surface stained with a silver stain Warthin-Starry. (Shah and Stosor 2001)

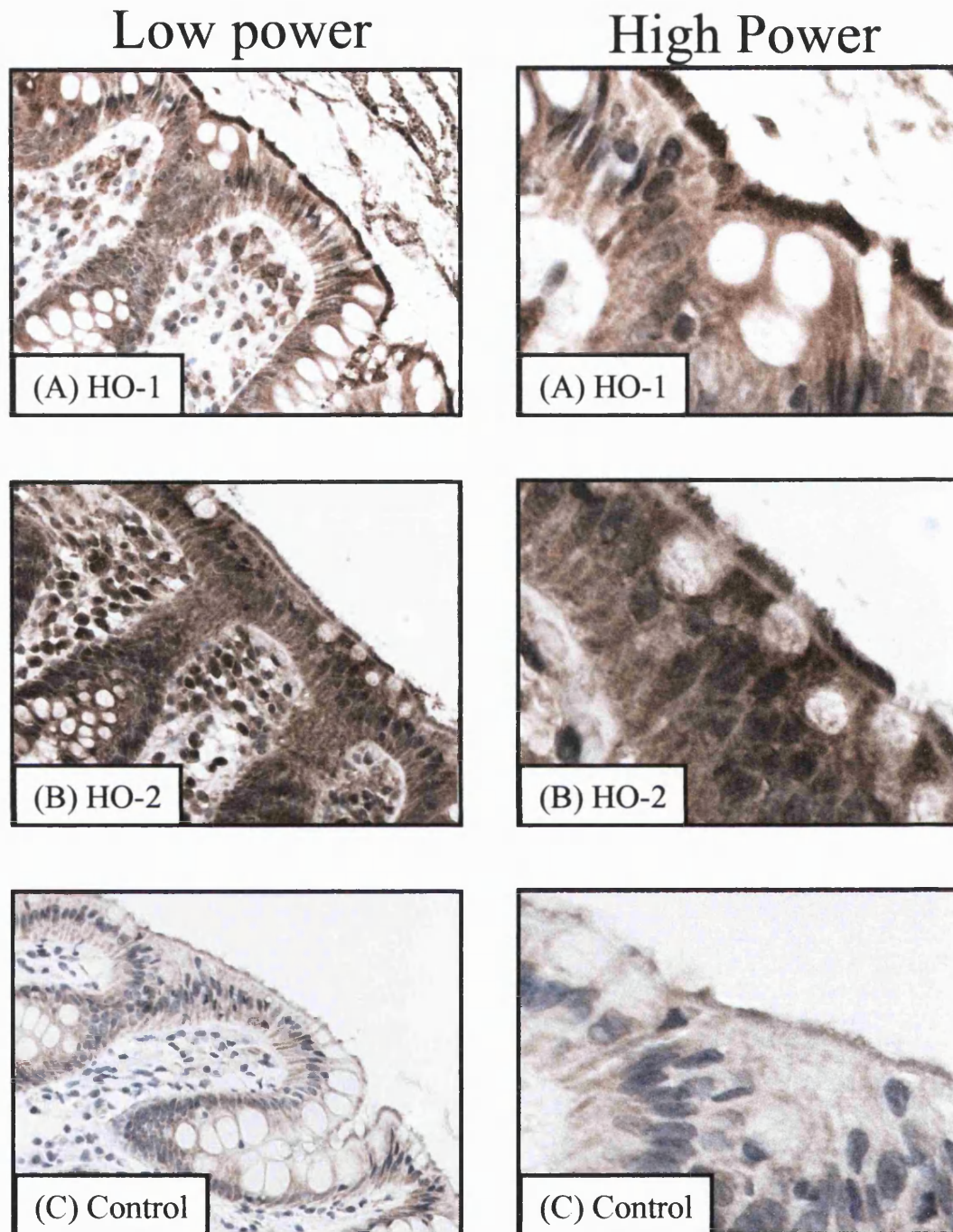


Figure 38 Spirochaetes express HO-1, demonstrated by immuno-staining.
 HO immuno-reactivity for (a) HO-1 and (b) HO-2 and (c) control in a mild case of IBD, (brown stain is positive for the HO protein). Spirochaetes are clearly visible on the epithelial surface and stain strongly suggesting HO-1 and HO-2 expression. Images on the left 128 x magnification and on the right 320 x original magnification. Full conditions described in Materials and Methods and summarised in Figure 10.

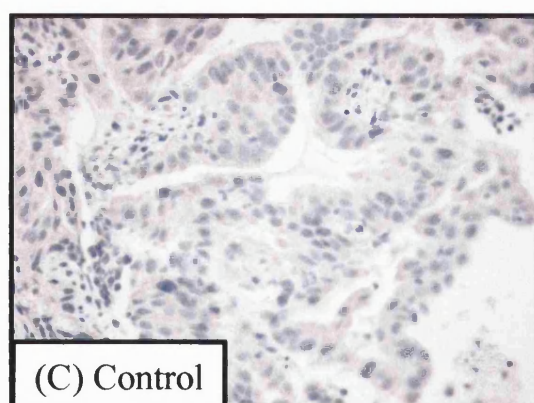
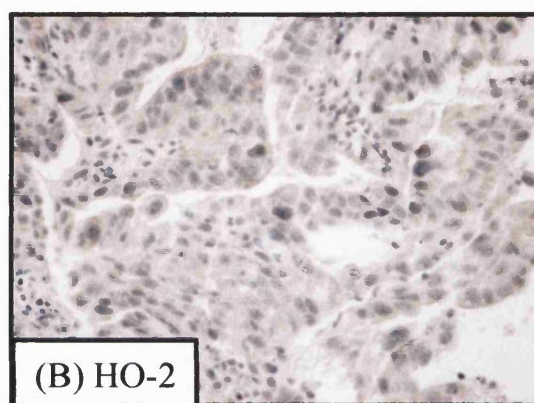
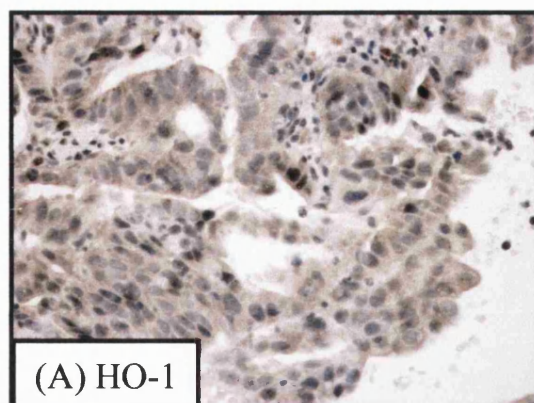


Figure 39 HO immuno-reactivity of a gastric carcinoma, demonstrating high expression of HO-1 especially in the epithelial cells. Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

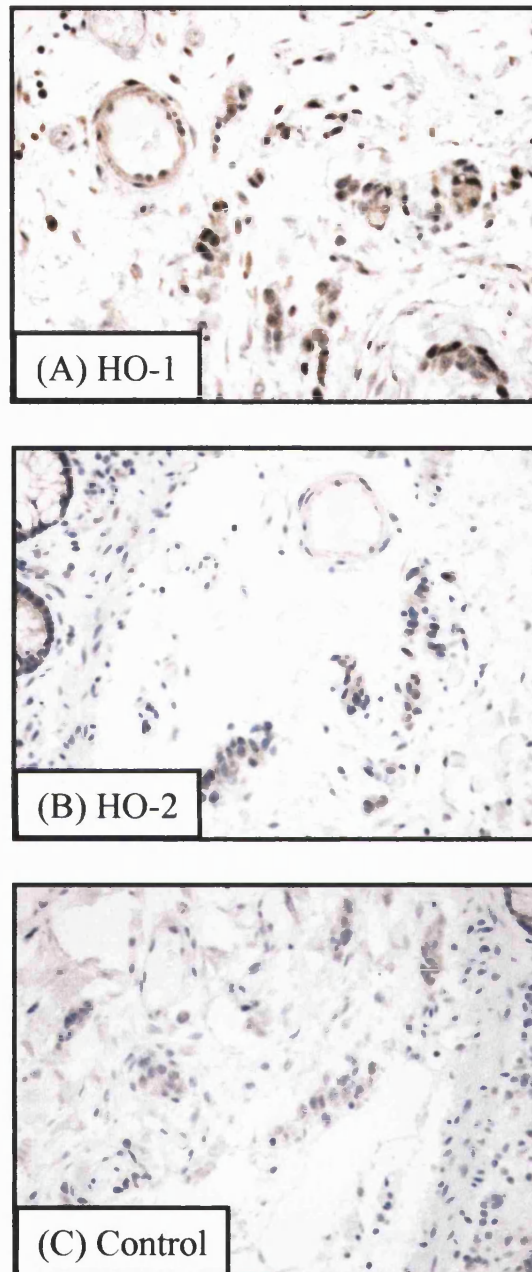


Figure 40 HO immuno-reactivity in a gastric cancer which had metastasized to the colon. The gastric carcinoma appears to express HO-1 particularly strongly. Original magnification 128x. Full conditions described in Materials and Methods and summarised in Figure 10.

3.9 Results summary

3.9.1 Immuno-staining for HO-1 and HO-2

- HO-1 is expressed in the terminal ileum and co-localises with HO-2.
- HO-1 and HO-2 are expressed in normal colonic mucosa in the resting state, both in the epithelial cell layer and in the inflammatory cells of the lamina propria.
- Expression of HO-1 occurs in the blood vessel walls and in the parasympathetic ganglia in the submucosa of the healthy colon.
- Expression of HO-1 increases in conditions of inflammation /cancer.

This increase was significant in IBD, CRC and Barrett's oesophagus.

	Lamina Propria HO-1 expression	Epithelium HO-1 expression
IBD	Increased	
CRC		Increased
Barrett's Oesophagus	Increased	Increased

- Immuno-staining in the epithelium of IBD and CRC and the lamina propria of CRC were also increased but did not reach statistical significance.
- Incidental finding that expression of HO-1 occurs in intestinal spirochaetes.

3.9.2 Summary of protein measurement in HT29 cell line model

- HO-1 expression is marginally increased after UVA treatment. This is similar to the known response of the skin fibroblast cell line FEK₄.
- HO-1 protein is expressed in HT29 cells within an hour of stimulation. (HO-1 is probably expressed in the un-stimulated state).
- The application of the cytokines TNF α and IL-13 had no great effect on the expression of HO-1 by HT29 cells. Basal expression of HO-1 was seen and not greatly changed by the action of these cytokines.

4 Discussion

This study demonstrates increased expression of HO-1 in IBD, colorectal carcinoma and Barrett's oesophagus when compared to normal healthy gut tissue. HO-1 and HO-2 are expressed in similar distributions. The increased expression of HO-1 may represent an appropriate host response to oxidant stress, whereby the expression of the inducible form of an anti-oxidant enzyme (HO-1) is increased to minimize tissue damage resulting from the presence of reactive oxygen species.

4.1 Normal Tissue Discussion

Immuno-staining confirmed the expression of HO-1 in both the lamina propria (predominantly in macrophages) and in the epithelial cell layer. The expression of HO-1 in normal colon and terminal ileum with no macroscopic features of inflammation is an interesting observation. Other research groups have demonstrated that in lung tissue there was little or no expression of HO-1 under resting conditions. However, after a period of hypoxia, macrophages migrate to the region of the pulmonary epithelium and are the first cells to express HO-1 (Carraway, Ghio et al. 2000). After a period of several weeks HO-1 expression is then seen in the pulmonary epithelial cells. The pattern in the colon would appear to be quite different. HO-1, the inducible form of the enzyme appears to be expressed even under resting conditions. This may reflect the nature of the inflammatory response in the GI tract. The GI tract is constantly exposed to a massive luminal antigen load and so may be thought to be in a constant state of controlled inflammation. Even in normal tissue the lamina propria has a

population of inflammatory cells including lymphocytes and macrophages. These inflammatory cells in the lamina propria act as sentinels of the host immune system, sampling antigenic material and raising the appropriate response.

In this work the macrophages were found to express HO-1. The identity of macrophages was determined by their characteristic morphology and the use of a macrophage specific immuno-stain α -CD68. A more elegant way to confirm this conclusion would be to double label the same slide. Images of HO-1 expression could then be generated before treatment to fully elute the HO-1 antibody then the same slide could be stained using α -CD68 antibody to identify macrophages. Co-localization could then be demonstrated by the superimposition of the two images.

4.2 IBD Discussion

In the IBD specimens studied there was a significant increase in expression of HO-1 in the lamina propria. There was also an increase in expression of HO-1 in the epithelial cell layer, although this did not reach statistical significance. The increase in HO-1 expression seen in the tissue of IBD colonic biopsies may represent a physiological response to increased oxidant stress in tissue undergoing inflammation. Macrophages have previously been shown to be a rich source of HO-1 in pulmonary tissue (Maestrelli, El Messlemani et al. 2001). Macrophages are a feature of inflammation of IBD; therefore it could be argued that it is not surprising that HO-1 is linked with inflammatory change. It is however significant that there is no evidence of a deficiency of HO-1. Thus there is no support for the notion that deficiency of HO-1 is a primary event in promoting inflammation in IBD.

4.3 Colorectal Carcinoma Discussion

In the colorectal cancers studied there was a significant increase in the expression of HO-1 in the epithelial cell layer when compared to normal colonic biopsies. These changes were also seen in a gastric carcinoma and in a gastric carcinoma, which had metastasized to the colon. (Figures 39, 40). Perhaps the increased expression of this anti-oxidant enzyme is a response to the greater cell turnover. Tumours are likely to generate oxidant stress, as cell growth may outstrip the vascular supply. However, it is possible that some of the observed differences in HO expression are related to the differences in morphology of the cells in the two types of tissue. HO-1 is expressed mainly in the cytoplasm of the epithelial cells. Colorectal carcinoma cells tend to be plump having relatively more cytoplasm (relative to the nuclear component of the cell) than normal cells. Even allowing for this, the observed intensities of staining for normal and colorectal carcinoma biopsies are strikingly different.

HO-2 (the constitutive form of the enzyme) was expressed in both normal tissue and that from colorectal cancers. As the data above suggests levels of HO-2 appear to be raised in the colorectal biopsies although this data was not analysed for statistical significance. The HO-1 and HO-2 antibodies used are designed not to cross react (see methods).

4.4 HO-1 protein expression by HT-29 cells

HO-1 expression was demonstrated by immuno-histochemistry in normal colonic epithelial cells. Western blotting confirmed that HO-1 protein was expressed in the resting state by the HT29 cell line and this observation is in keeping with the immuno-staining work. However, it is possible that the culture conditions endured by the cell line may expose the cells to continuous oxidative stress. The in vitro preparation may therefore express HO-1 as a response to the culture conditions. It must also be remembered that the HT-29 cell line is derived from a primary colonic tumour and not from normal colonic epithelial cells. UVA stimulation could marginally increase HO-1 protein expression by HT-29 cells however stimulation with IFN γ and TNF α did not increase HO-1 expression above basal levels.

4.5 Barrett's Oesophagus Discussion

Immuno-staining showed HO-1 expression is increased in both the lamina propria and the epithelial cell layer in Barrett's oesophagus when compared to normal oesophageal biopsies. So a lack of HO-1 and the resulting anti-inflammatory/anti-oxidant influence is not a causal factor in the inflammatory processes leading to the development of Barrett's oesophagus. Rather it would appear that in the presence of Barrett's oesophagus the expression of HO-1 is increased. This may represent an appropriate physiological response to the inflammation with the change in HO-1 expression appearing to be a secondary event. The increase in expression of HO-1 occurs in both the lamina propria and the epithelium.

4.5 HO expression by intestinal spirochaetes

Parr first described Spirochaete colonisation of the gut in 1923. The term intestinal spirochaetosis was introduced by in 1967 by Lee et al. By using electron microscopy they visualised a forest of organisms adherent to the epithelial surface of the rectum. Some organisms imaged tangentially showed spiral filaments within their cytoplasm, confirming that they were spirochaetes. Conventional light microscopy of H + E slides can also detect spirochaetosis, appearing as a false brush border on the epithelial surface. This hazy blue border is made up of many long thin spirochaete organisms attached end on to the epithelial surface with no evidence of invasion (Figure 37a, Shah 2001). Silver staining demonstrates intestinal spirochaetes extremely clearly (Figure 37b). The mucosa appears normal at colonoscopy, intestinal spirochaetosis being usually an incidental histological finding.

Intestinal spirochaetosis occurs in the faeces in 1.2 % of healthy individuals and in hospital patients undergoing rectal biopsy the rate of spirochaetosis ranges from 1.9 % to 6.9% in the U.K. (Takeuchi 1974, Lee 1971). Prevalence rates vary in different countries and it is thought, for example, to affect almost the entire population in West Africa. High rates have also been reported in homosexual men who have a history of anal intercourse (28 to 36 %, Abrams 1998). At least two species of spirochaetes have been implicated; *Brachyspira pilosicoli* and *Brachyspira aalborgi* (Rodgers 1986, Nielson 1983). They vary in length from 0.5 to 20 μm . They are difficult to grow in culture.

The pathological significance of intestinal spirochaetosis remains uncertain. Many clinicians believe that intestinal spirochaetosis is not associated with GI disease and therefore elect not to treat it. This strategy is supported by the observations that intestinal spirochaetosis can be present without symptoms, with no obvious associated inflammatory response and with no invasion of the epithelium. One interventional study showed a rate of intestinal spirochaetosis of 5% in 300 patients who underwent sigmoidoscopy (Neilson 1983). Treatment with neomycin did not affect symptoms. However, there have been a series of case reports that speculate that chronic infection may cause symptoms of abdominal pain and diarrhoea with or without blood. (Heine 2001). Almost invariably such case-reports describe relief of symptoms following anti-microbial therapy with metronidazole or neomycin. Generally such case reports include only a handful of patients and arguably represent a positive reporting bias. Improvement of symptoms after treatment with such broad-spectrum antibiotics may represent an effect independent of spirochaetosis.

In two of the colonic biopsies immuno-stained for the HO proteins there was coincidental spirochaetosis. In both cases the spirochaetes stained heavily for both HO-1 and HO-2 as demonstrated by the observed heavy brown staining Figure 34. High levels of expression of HO-1 may represent an evolutionary mechanism developed to protect these bacteria from the hostile conditions of oxidative stress within the gut lumen. Other bacteria have developed similar strategies to cope with life in the GI tract. For example, *Helicobacter pylori* employs a urease

enzyme to create a local alkaline solution to buffer the acidic conditions present in the stomach.

4.6 The role of HO-1 in inflammatory conditions in the GI tract

HO-1 may offer a cytoprotective role within the GI tract. As described in the introduction this enzyme has been retained avidly throughout evolution; the structure has 90% homology between the rat and human species. HO-1 occurs in similar forms across many organisms and many cell types. The metabolism of heme could have been localised to a small region of the organism, with the enzyme restricted to that organ. The observation that the HO-1 enzyme has been retained and can be widely expressed across many cell types may suggest that it has a broader cytoprotective function. If HO-1 functions as an inducible antioxidant enzyme with cytoprotective actions it is not surprising that it should be induced in states of inflammation or increased cellular turnover such as IBD, colorectal carcinoma and Barrett's oesophagus.

Since the experimental work in this thesis was conducted a small study looking at HO-1 in gastritis and a small number of cases of colitis has been published (Barton 2003). Although this work only looked at biopsy specimens it broadly supports the observation that HO-1 is present in normal GI mucosa and increased in areas of inflammation in both colitis and helicobacter pylori related gastritis. No other work related to HO-1 expression in colorectal cancer or Barrett's oesophagus has yet been published.

The anti-oxidant influence of HO-1 can be accounted for by considering the result of the metabolism of heme to form CO, free iron (Fe^{2+}) and biliverdin. Heme is toxic and its metabolites have anti-oxidant properties. Perhaps the most important of the products is CO, which is increasingly being recognised as a major secondary messenger that at low concentrations can account for many of the actions of HO-1. Free iron in the ferrous state (Fe^{2+}) not only leads to the production of free radicals via the Fenton reaction but it also causes increased production of ferritin, which chelates the ferrous iron and is itself an anti-oxidant. Biliverdin and its metabolite bilirubin both act as anti-oxidants and free radical scavengers.

4.6.1 Is HO-1 always cytoprotective?

In other systems, notably in the lung, induction of HO-1 has been shown to be associated with oxidant injury. HO-1 is generally thought to offer a cytoprotective influence. This protective effect of HO-1 is supported by the observations that inhibition of HO-1 worsens injury (Wang 2001), treatment with an adenoviral vector capable of HO-1 production lessens injury (Haider 2002) and that CO one of the products of HO-1 metabolism of heme is cytoprotective at low concentrations (Otterbein 2000). However there is some evidence that HO-1 may not always have a cytoprotective action: there may be a threshold effect whereby very high levels of the HO-1 enzyme products can lead to tissue damage (Suttner 1999). It may be that overall HO activity is crucial rather than simply that of HO-1 alone. Weng's group has shown that in tissues where both HO-1 and HO-2 co-localise such as the brain HO-1 interacts with HO-2 and the HO-1 HO-2 complex has a lower enzymatic activity than the expected sum of each enzyme (Weng

2003). It has been suggested that HO-1 binds to HO-2 in such a way as to obscure the binding site for heme. The co-localisation seen in the gut may serve to keep overall HO enzyme activity within a range that is cytoprotective. This may be important in the GI tract. In the examples considered in this study there is evidence for very high levels of HO-1 expression in the lamina propria of IBD, the epithelial layer of colorectal cancer and in both layers in Barrett's oesophagus, an interaction between HO-1 and HO-2 must be considered.

The relative lack of expression in normal oesophagus may imply that HO-1 production is a feature of columnar epithelial cell function (seen in normal colon, IBD, colorectal carcinoma and Barrett's oesophagus but absent from normal oesophageal epithelium which is of a squamous type). This may account for the very low levels of HO-1 expression found in the normal squamous epithelium of the oesophagus. Another explanation of the lack of HO-1 in the oesophagus may be related to antigen exposure. The oesophageal epithelium is relatively unchallenged by luminal antigens. There is rapid transit of the undigested food bolus through the oesophagus, perhaps this accounts for the much-reduced HO-1 response in this region compared to more distal parts of the GI tract.

4.6.2 Limitations of the interpretation of Immuno-staining

The immuno-staining technique can determine the distribution of the enzyme in the tissue. This technique can identify which cell types express the enzyme (see future work on immuno-fluorescence and co-localisation). The density of staining can be used to draw semi-quantitative conclusions regarding HO-1 expression.

Thus in the present work it could be used to indicate that HO-1 expression is increased in IBD, colorectal carcinoma and Barrett's oesophagus.

There are some inherent problems encountered in interpreting the results of immuno-staining because other variables can influence the darkness of the staining. These include: concentration of the primary and secondary antibodies; incubation time; temperature; level of endogenous peroxidases in the tissue. This study attempted to control for these variables by standardising the processing of the slides, and by processing them in mixed batches that included normal and diseased tissue.

4.6.3 Inter-observer variation in scoring the immuno-staining results

There was only a moderate degree of agreement between the two independent observers (MF and NR) with only 78% of the cases being scored the same. These were truly independent assessments carried out without coaching or involving practice cases. The agreement in future might be improved by further clarification of the grading system.

4.6.4 HO-1 enzyme activity

The influence of HO-1 on oxidant stress will depend not only on the level of expression of the enzyme but also the activity of the enzyme. Enzyme activity has not been measured in the present study. Although this study indicates increased expression of HO-1 in IBD, colorectal carcinoma and Barrett's oesophagus it is possible that the enzyme activity may also be altered in these disease states. If the enzyme activity were greatly reduced in these disease states then there could be a

relative lack of anti-oxidant activity. If HO-1 activity is normal in these diseases then the increased expression would amount to an appropriate physiological response to the increased oxidant stress produced by the disease, not a causal event in the disease processes as suggested in the original hypothesis.

If we make the reasonable assumption that the increased expression reflects increased enzyme activity then HO-1 of normal activity is expressed in increased quantities as a response to the oxidant stress generated by IBD, colorectal carcinoma and Barrett's oesophagus. Therefore the drive for the inflammation is not related to a deficiency of HO-1. Oxidant stress, however, remains an important influence in conditions characterised by ongoing inflammation and so manipulation of HO-1 may still prove to have therapeutic potential.

4.7 Future work

The present study has opened up and suggested the possibility of future work that could refine and extend our knowledge of the relevance of HO-1 in IBD, colorectal carcinoma and Barrett's oesophagus. Several of these possibilities are outline below.

4.7.1 Developments of immuno-staining: co-localisation experiments

In the present study consecutive slices from the same block of bowel tissue were used to compare the distribution of different proteins in the sample. The first slice was stained for the expression of HO-1 and the next for the expression of HO-2. Having cut through the same architectural features an approximate comparison of the expression of the two enzymes could then be made. A more elegant way to investigate the patterns of expression of HO-1 and HO-2 would be to use the standard immuno-fluorescence with FITC (fluorescein isothiocyanate) conjugated to the secondary antibody. A photograph of the first primary antibody result would be taken under the appropriate lighting. Then after soaking off the slide cover and treating with KMnO_4 in H_2SO_4 to remove the HO-1 antibody complex, the fluoresciene staining could be repeated with a different primary antibody to detect HO-2. The two different proteins of interest could then be visualised as different colours and superimposed on the same image to show their differing distributions within the same specimen. This technique could also be used to compare the expression of HO-1 and also the distribution of macrophages (using the α -CD68 antibody). This technique was used by (Donat 1999) to show that in the rat ileum a small subset of ganglion cells displayed both HO-2 and NOS immuno-reactivity.

4.7.2 Further analysis of IBD immuno-staining

It would be interesting to extend this work to a larger number of cases with IBD. With a larger data set it would be possible to verify the result that HO-1 expression is increased in the lamina propria of IBD and also to explore the trend of increased expression in the epithelial cell layer in IBD. It would be interesting to subdivide the cases into different disease groups: Crohn's and ulcerative colitis. The inflammatory changes affect the full thickness of the bowel wall in Crohn's disease but are much more superficial in UC. It would also be desirable to investigate whether the degree of inflammation (assessed by histological features) within the biopsy correlated with the expression of HO-1: this would be best assessed by analysing a series of full thickness sections gained at colectomy for IBD. By extending the results from this study it might be expected to establish that increased HO-1 expression is associated with inflammation with HO-1 levels being highest in the most inflamed specimens.

The effects of drugs on the expression of HO-1 remain unclear; all future cases examined should have a full drug history taken including details of exposure to corticosteroids, 5 amino-salicylates and non-steroidal anti-inflammatory agents (Lavrovsky 1996). In IBD cases it will also be possible to compare samples from the same subject at the time of active macroscopic inflammation and during remission, and also from areas of the colon that are inflamed and from areas that macroscopically appear normal.

4.7.3 Culture of primary epithelial cells

Work on primary cell collection and culture has begun, but will need significant refinement to achieve a model in which primary cell would survive in culture for at least 24 hours. To investigate and test the findings suggested by immunostaining further quantitative analysis of HO-1 should be undertaken comparing diseased and normal tissue. It would be important to assess HO-1 protein expression in the un-stimulated colon primary cells. It would also be possible to measure the production of HO-1 mRNA using real-time PCR.

4.7.4 Extend the study of HO-1 expression by Intestinal Spirochaetes

Future work would also study in greater detail the expression of HO-1 by intestinal spirochaetes, to validate the expression of HO-1 seen in two cases in the present study. To do this a prospective program of specimen collection would have to be undertaken. Because of the new legislation (*Human Tissue Bill*) regarding the use of historically held histopathology slides, in the future it will only be possible to use stored specimens for research if the patient has granted specific permission for this purpose before biopsy was taken. Although intestinal spirochaetosis is relatively common, with a prevalence of approximately 1%, it often goes unreported as it is thought to have little pathological significance. Therefore an active program of case finding would have to be undertaken.

4.7.5 Does HO-1 modulate anti-inflammatory mediators in the gut?

Given my hypothesis that HO-1 mediates anti-inflammatory effects in the gut, the effect of pharmacological manipulation of HO-1 on basal and cytokine-stimulated

production of pro-inflammatory mediators such as COX-2 and NOS2 could be assessed. HO-1 will be selectively stimulated using heme. The role of HO-1 in this system could further be investigated by employing its selective inhibitor zinc protoporphyrin. Another way to explore the influence of HO-1 on colonic epithelial cells would be to incubate the cells in an environment with low levels of CO. CO may have anti-inflammatory influence by up-regulation of IL-10 and down-regulation of TNF α and IL-1 (Minamino 2001)(similar effects are seen in macrophages exposed to LPS). It is also possible that the pro-inflammatory cytokines may stimulate expression of HO-1 as part of a negative feedback loop to limit tissue damage caused by local inflammation.

4.7.6 Measuring HO-1 enzyme activity

To investigate the role of HO-1 in the inflammatory response, measuring functional enzyme activity is more important than measuring enzyme expression. To assess HO-1 enzyme activity a variation on a technique described by Carraway could be used (Carraway 2000). The homogenised portion of gut tissue would be placed in phosphate buffer with MgCl₂ then sonicated and centrifuged to liberate HO enzymes into the supernatant. Immuno-precipitation with an antibody specific to HO-1 could be used to separate this isoform of the enzyme from the constitutive forms (HO-2 and HO-3). The immuno-precipitate of HO-1 would then be incubated with liver cytosol (acting as a source of bilirubin-reductase) with a fixed amount of heme, NADPH, glucose-6-phosphate and G6P-dehydrogenase. The amount of bilirubin produced could then be assessed using a spectrophotometric analysis to measure the colour change consequent on the production of bilirubin.

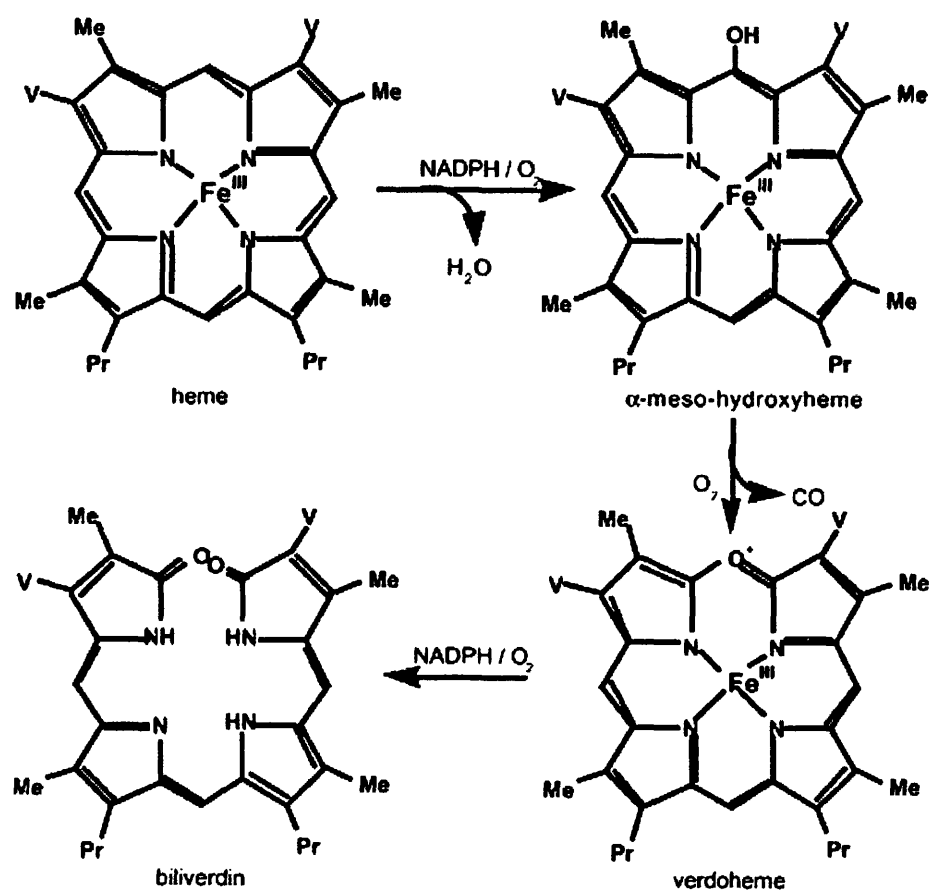
The amount of bilirubin produced would then give a measure of HO-1 enzyme activity. In view of the suggestion by Weng that co-localisation of HO-1 and HO-2 may result in a complex of the two enzymes with a lower enzyme activity than the sum of the two enzymes, it would also be important to calculate total HO activity.

4.7.7 Levels of CO on flatus as a marker of HO-1 activity

In the respiratory system an increase in exhaled CO produced by the action of HO-1 has been found in acute asthma. It has been suggested that the measurement of exhaled CO might predict relapse and offer a marker of disease progression in lung disease (Zayasu 1997). It would be interesting to analyse flatus for CO to see if levels vary in inflammatory conditions affecting the colon. This could help assess whether a change in bowel symptoms was related to a flare of IBD. Changes would however be rather non-specific, since this study has shown that HO-1 is increased in a number of different disease states both inflammatory and cancer related.

Conclusion

The original hypothesis suggested that a deficiency of the anti-oxidant enzyme HO-1 could account for some of the inflammatory features associated with IBD, colorectal carcinoma and Barrett's oesophagus. The finding in this study that increased levels of HO-1 are present in tissue from such disease states indicates that a deficiency of HO-1 is unlikely to be a causal event in the pathogenesis of IBD, colorectal carcinoma and Barrett's oesophagus. Rather, in these diseases there appears to be an appropriate physiological response to increased oxidant stress that involves the production of the inducible anti-oxidant enzyme HO-1.



Appendix I The reaction intermediates of heme breakdown

Appendix II

Patient information sheet for bowel inflammation study

Introduction

You have been invited to take part in a research study. This information sheet is designed to help you to understand what the research is about and what it involves. Please take some time to read it and discuss it with your hospital doctor. Ask about anything that is not clear or if you need more information. Take time to decide whether you would like to take part or not.

Thank you for reading this.

Purpose of this study

In this department we have a special interest into what controls inflammation within the human gut. Failure of control of inflammation causes conditions such as inflammatory bowel disease and is known to increase the risk of cancer. Much of the work in this area has been performed on abnormal cells and animal specimens. To understand exactly what happens in the human gut it is important to check this work in tissue from people rather than animals.

Why have I been chosen?

You have been chosen for this study because you are due to have a portion of your gut removed at surgery. We would like to take a small part of the bowel that will be removed. The size of the piece of the bowel that will be removed will be exactly the same regardless of whether you take part in the study or not. We will extract cells from this piece of gut and over a period of 3 weeks perform experiments to study inflammation. In total we plan to study about 60 patients in this way.

Do I have to take part?

It is up to you to decide whether to take part. If you do decide to take part you will be given this information sheet to keep and will be asked to sign a consent form. If you decide to take part you will be free to withdraw at any stage and do not need to explain your reasons for doing so. This will not affect the standard of care that you will receive.

What happens if I decide to take part?

If you take part in this study there will be no difference in anyway in your operation or your care. The only sample that we require is part of the gut that would have been removed any way at your operation.

Appendix II

What do I have to do?

This study only requires you to give us permission to keep some of the tissue removed at your operation. The tissue will be kept for up to 3 weeks and then all the cells will be completely destroyed. You will not need to do anything else and being part of the study will not affect your treatment in any way.

What are the possible disadvantages of taking part?

As this study does not change your treatment in any way there are no disadvantages of taking part.

What are the possible benefits of taking part?

There will be no direct benefits to you. The information the doctors discover as a result of this study may help them understand why abnormal inflammation causes disease in some people. This may help treat other patients in the future.

Will my taking part in this study be kept confidential?

The study doctors will need to access your medical records to check your diagnosis. This information will only be available to the researchers directly involved in the study. Your results will be kept strictly confidential and any information that leaves the hospital will have your name and address removed so that you cannot be identified.

What happens to the results if this study?

When we have enough results we will publish the work in a medical journal for other doctors to examine. By this method gradually we gain a greater understanding of diseases and how to treat them. None of the participants will be identified in any report.

Who is organising and funding the research?

One of the consultant gastroenterologists and his team together with a scientist at the University of Bath are organising the research. This research project is being sponsored by the Wellcome Trust. None of the doctors or scientists are being paid to recruit patients.

Who has reviewed the study?

This study has been reviewed by the Bath local research ethics committee.

Contact for further information?

Dr DA Robertson, Consultant gastroenterologist, Royal United Hospital, Bath
Dr MA Feeney, Research Fellow, Royal United Hospital, Bath
Tel 01225 428331 Ext 4547

Appendix III**Oesophageal subjects**

	Normal Oesophagus	Barrett's Oesophagus
n	20	18
Average Age	62yr	69yr
Male (%)	12/20	15/18
Female(%)	8/20	3/18

Colon subjects

	Normal Colon	CRC	IBD
n	28	16	22
Average Age	61yr	76yr	47yr
Male (%)	14/28	11/16	8/22
Female (%)	14/28	5/16	14/22

IBD analysed by disease type and activity

UC	12
CD	10
Mild inflammation	6/22
Moderate inflammation	14/22
Severe inflammation	4/22

Appendix IV, Quartile values of HO-1 immuno-staining

		Normal	IBD	CRC
Epi	Median	2	2	2
	25 th	1	2	2
	75 th	2	2	2
LP	Median	2	2	2
	25 th	1	2	2
	75 th	2	2	2

Oesophageal HO-1 immuno-staining scores

		Normal	BO
Epi	Median	1	2
	25 th	1	2
	75 th	2	2
LP	Median	1	2
	25 th	1	2
	75 th	2	2

Colon Control scores

	Normal	IBD	CRC
Median	0	0	0
25 th	0	0	0
75 th	0	0	1

Oesophageal control scores

	Normal	BO
Median	0	0
25 th	0	0
75 th	0	0

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